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in Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

Adenosine is a potential novel factor in breast cancer progression and this project has been designed to evaluate local adenosine tumor-promoting activity experimentally. We have aimed at testing growth and angiogenic properties of cells that differ in adenosine production rates in xenograft model using immunocompromized mice. During the 3-year period we have accomplished several specific goals successfully. We have generated cell variants that differ in their capacity to produce endogenous adensine. We have determined that that their growth rate as xenografts depends on the number of cells injected orthotopically into mice. At 2.5 x 106 cells per site we have seen decreased growth of eN(-) cells. In addition, we have demonstrated that eN specifically interacts with Tenascin C and cells with suppressed eN expression have enhanced motility on Tenascin C. Furthermore, using protein expression profiling we have determined that high eN expression coincides with mesenchymal phenotype and that HDAC inhibitor TSA reverses epithelial expression profile in breast cancer cells. However some goals have not been accomplished within the proposed time frame. Given the importance of proposed research we are continuing our work toward accomplishing these goals using other funding sources.

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Table of Content

1. Introduction

High expression of ecto-5'-nucleotidase (eN) in advanced breast carcinoma leads to locally high concentration of signaling adenosine. Since adenosine has potent angiogenic, growth-stimulatory and immunosuppressive activities in other tissues, we raised the question whether similar functions of this regulatory molecule would apply to breast cancer (reviewed in (1). This project has been designed to test this hypothesis and ask whether increased/decreased levels of adenosine in breast cancer may lead to acceleration/inhibition, respectively, of tumor progression in mice xenograft model.

The key enzymatic activity that produces pharmacologically active adenosine in epithelial cells is ecto-5'-nucleotidase (eN). We found that eN, that is attached to the extracellular face of cell through the GPI anchor, is highly expressed in ER(-) breast cancer cells and clinical samples (2). We also found that expression of eN and another adenosine-regulating protein adenosine deaminase (ADA) is regulated by β-catenin, PKCα and estrogen receptor, further suggesting that increased or decreased expression of these proteins during breast cancer may lead to increased adenosine generation.

Thus, these data and the wealth of published observations on the physiological functions of adenosine in other tissues strongly suggest that eN and adenosine may constitute novel regulatory factors that may play an important role in breast cancer progression. If confirmed, both the eN directly and adenosine pharmacological activities indirectly may become targets for the development of new therapeutic agents.

2. Body of the Report
A. First year (summary)
Development of genetically altered breast cancer cell lines.

The aim of first year funding period was to develop and characterize several cell lines for use in this project. We used retroviral expression system to introduce both sense and antisense cDNAs of EGFP, ADA, mutated ADA and eN, respectively. We have performed preliminary expression profiling of selected clones to determine the stability of phenotypes. We have also confirmed the decreased rate of adenosine generation in cell clones that expressed low level of ecto-5'-nucleotidase (eN). We have observed some genetic instability of engineered clones, especially those expressing low eN and this problem was addressed during second year of funding period.

B. Second year (summary)
Maintaining and characterization of genetically modified breast cancer cell lines. Initial inoculation into nude mice.

The MDA-MB-231 clones were further characterized and evaluated for inoculations into nude mice. After development of cells that stably downregulated eN (eN_{low}) and overexpressed EGFP (green fluorescent protein, clones H2 and F4), ADAw/t (clones G11 and H10) and ADAmut, we observed a significant loss of effectiveness of suppression of expression of eN but not ADA and EGFP. To solve this problem we performed two additional rounds of clonal selection of previously selected eN_{low} clones to select subclones that were more stable in long term culture. This strategy was successful and we have finally obtained three eN_{low} MDA-MB-231 clones that were further characterized (eN_{low} clones G12, G3 and H6). We were not successful in obtaining stable MCF-7, BT474 and ZR-75-1 clones with high expression of eN.

To characterize MDA-MB-231 cell clones and other human breast cancer cell lines in detail we compared the expression of 114 membrane and cytoskeletal proteins in 15 cell lines. This profiling confirmed that breast cancer cell line that have high potential to generate extracellular adenosine all exhibit mesenchymal phenotype (Appendice D). Among altered MDA-MB-231 cells we found no major shift in expression profile that would suggest altered phenotype inadvertently caused by genetic manipulations not related to the expression of ADA, EGFP and eN *per se*. However, we found altered adhesiveness of MDA-MB-231 clone with low eN to Tenascin C. Thus these experiments demonstrate for the first time a specific interaction between eN and Tenascin C and identified two-way functional relationship between these proteins: eN as a receptor for Tenascin C and Tenascin C as an inhibitor of eN and adenosine generation. This discovery has been an exciting new development that further validate our hypothesis on the important functions of eN and adenosine in breast cancer progression.

Initial inoculations of modified MDA-MB-231 cell in nude mice.

We have utilized 9 mice to test if inoculation of 2.5 x10^6 cells per site will be sufficient to develop grafts of sufficient size for further analysis. In this experiment we used EGFP$_{high}$-1 (clone H2)cells as controls and ADA$_{high}$-1 (clone G11) and eN$_{low}$-1 (clone 6H) cells to manipulate tumor adenosine concentrations. Results shown in Fig. 7 demonstrate that while grafts ADA$_{high}$-1 grew as controls, eN$_{low}$-1 cells developed similarly sized tumors with significant delay. This result provides the first strong argument supporting the role of adenosine and/or eN in breast cancer growth. Currently, we are in a process of setting up a major experiment that will test two cell lines of each type and analyze the angiogenic and endothelial parameters by immunohistological and biochemical means.

C. Third year
Further analysis of MDA-MB-231 cell variants in nude mice and continuation of analysis of interaction of eN with Tenascin C.

Our focus during third and last year of granting period was on obtaining definitive results on the potential discriminating role of eN and/or adenosine in breast cancer growth and progression. Furthermore, our observation that eN expression coincides with mesenchymal phenotype in breast cancer cells, we aimed at investigating the effect of HDAC inhibitor TSA, a differentiating agent, on the expression of eN and other mesenchymal markers. Finally, our discovery that eN interacts with Tenascin C prompted us to definitively determine whether this interaction is observed with partially purified proteins. Although two last objectives go a little bit beyond proposed research plan, they appear to be increasingly critical for the in-depth evaluation of the role of eN and/or adenosine in breast cancer progression.

C.1. Inoculation of developed cell lines into mause mammary fat pads
Preliminary results obtained during first inoculation experiments suggested that low expression of eN may play important role in tumor growth. Since the xenograft growth rate was relatively slow in first experiment, we have increased the number of inoculated cells to 6x10^6 per site, expecting the much faster growth rate of grafts. Unfortunately, as shown in Fig. 2, the growth rate increased slightly, however all cell variants grew at the similar rate and no differences were observed. In the follow-up experiment we have decided to further evaluate this cell number dependence of tumor growth and orthotopically injected 2.0 x 10^6 cell per site. This particular time we have not seen any tumor growth. In each of this cases we have closely monitored the expression levels of eN, EGFP, ADA, CD44, Integrin β1 and EGFR and have not observed significant changes in expression profiles. These results suggest that, in the absence of changes in expression of other proteins, the growth of xenografts in nude mice strongly depend on the number of injected cells. This has significantly delayed
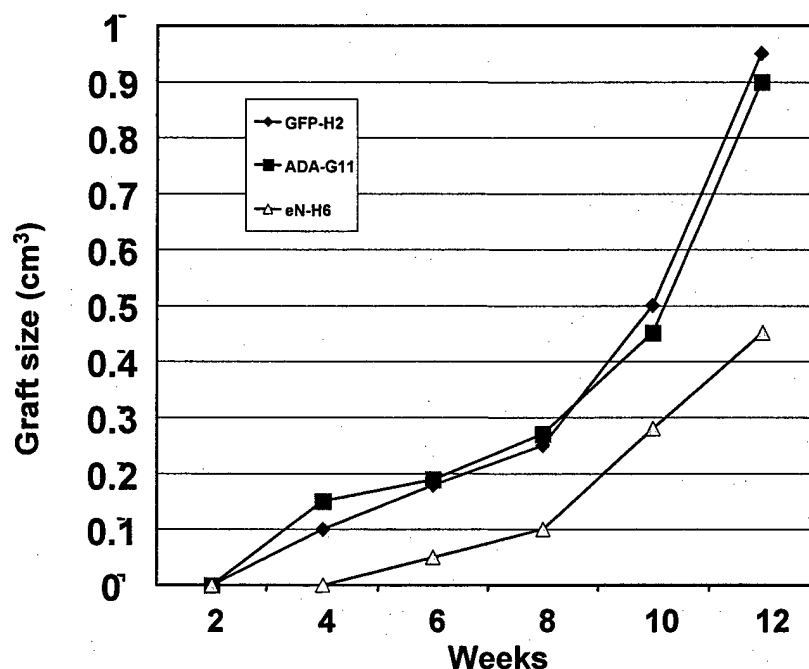
Fig. 1. The growth of orthotopically grafted cells GFP-H2, ADA-G11 and eN-H6 (eN$_{low}$) in nude mice. Graft sizes were measured weekly or more often and two-weeks measurements are shown in cm^3. Each point represents three independent grafts.
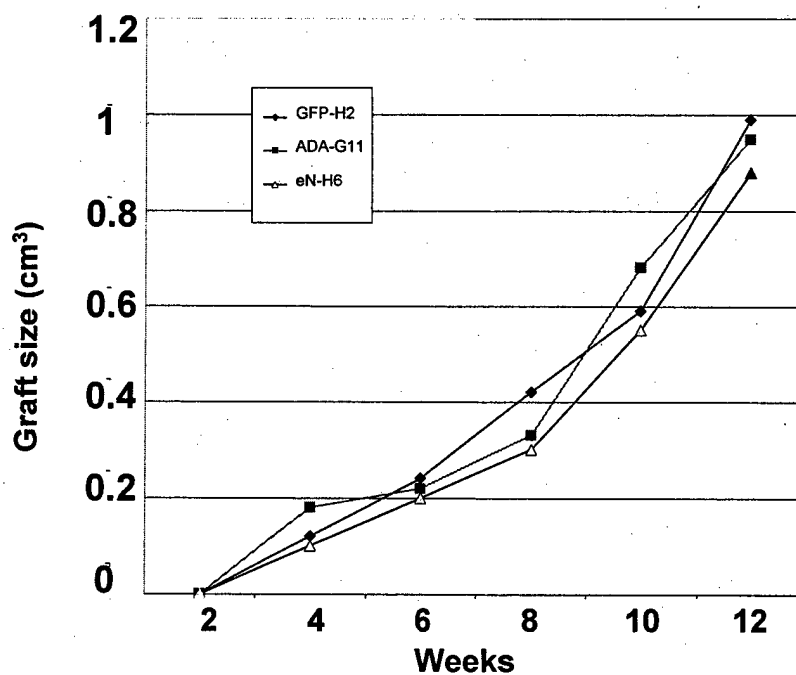

Fig. 2. The growth of orthotopically grafted cells GFP-H2, ADA-G11 and eN-H6 (eN$_{low}$) in nude mice. Graft sizes were measured weekly or more often and measurements are shown in cm^3. Each point represents five independent grafts.

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progress of this project. Currently, we are in a process of another experiments using cell number similar to that used in the first experiment.

C.2. Defining the interaction of eN with Tenascin C.

In meantime, we have further advanced our understanding of interactions of eN with Tenascin C. Previously, we have determined that decreasing the eN expression in MDA-MB-231 cells leads to a weaker adhesion to substratum covered with Tenascin C. We have used these two cell clones to determine whether the difference in adhesion would affect their motile properties. For this purpose we employed video microscopy. Data shown in Fig. 3 show that indeed, cell with low eN do migrate faster. Furthermore, to determine whether eN directly interacts with Tenascin C we have immobilized several ECM components on the column and used them with partially purified eN in column binding assay. eN was partially purified using MDA-MB-231 cell extract and ConA-sepharose. Eluted eN was divided into three equal portion, applied onto three separate columns, Tenascin C, Fibronectin and Collagen and eluted with increasing concentration of salt (Fig.4). Results indicate that eN was retained in column with immobilized Tenascin C and only small portion of eN was eluted during washing step. Much weaker interaction was with Fibronectin and no interaction was observed with immobilized Collagen. We conclude that eN specifically and strongly interacts with Tenascin C in vitro.

C.2. Role of HDAC inhibition in expression of eN in invasive breast cancer cells.

Inhibitors of histone deacetylases (HDAC), including TSA, have potent effects on global gene expression, inhibit cell proliferation and induce differentiation and apoptosis. Although HDAC inhibitors caused changes in many different cell types, mesenchymal cells were proposed to be particularly sensitive. In particular, previous studies have shown that MDA-MB-231 cells induced the expression of E-cadherin and ERα after treatment with HDAC inhibitor TSA. Given the known genome-wide effects of HDAC inhibitors, such response suggested that TSA may have induced differentiation, which in the context of mesenchymal cells may be regarded as mesenchymal to epithelial transdifferentiation (MET). Indeed, the expression of most markers that differentiate ER(-) and ER(+) cells were affected in a reciprocal manner Fig. 5). Thus, our expression profiling of a broader set of membrane and cytoskeletal proteins provide a strong evidence for MET after treatment of breast cancer cells of mesenchymal phenotype with TSA. In the context of eN and adenosine receptor function (Fig. 6), our result show that global expression change upon TSA treatment is associated with decreased potential for adenosine generation and altered signaling through adenosine receptors. Previous studies have shown that histone deacetylase inhibitors increased the expression of metastasis suppressors such as breast metastasis suppressor 1 (BRMS1), tissue inhibitor of metalloproteinase 3 (TIMP3) and nm23 and thereby may have therapeutic activity in metastatic phase of breast and other carcinomas. In this context our results
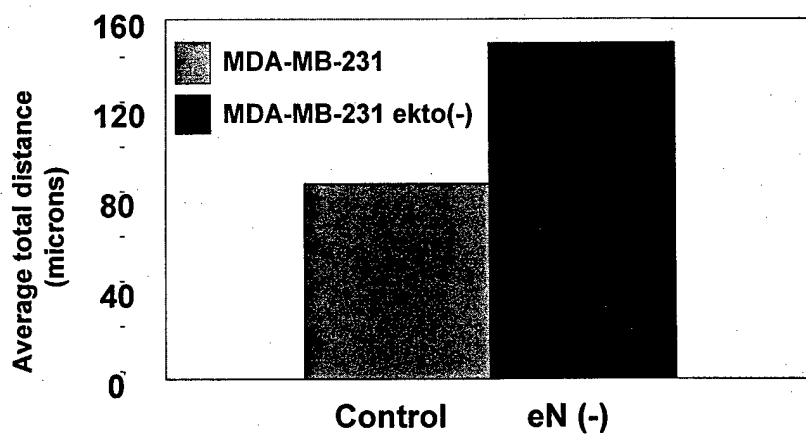
Fig. 3. Migration of control MDA-MB-231cells and clone G-12 with decreased eN expression on Tenascin C. Experiment was performed using video microscopy.
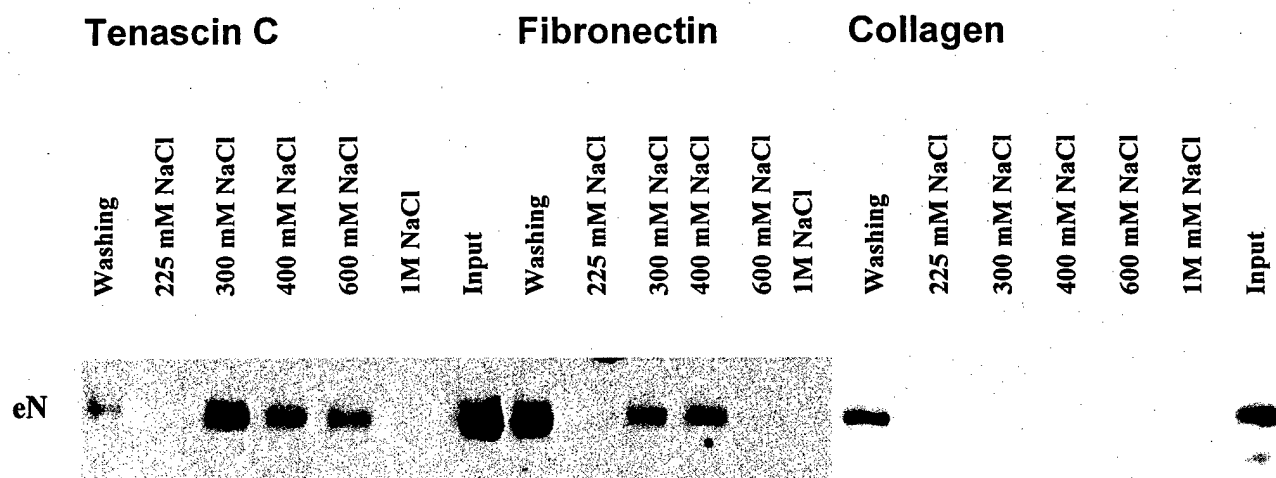

Fig. 4. Interaction of partially purified eN with immobilized Tenascin C and other ECM proteins. Input represent material that was loaded on immobilized Tenascin C, Fibronectin and Collagen columns. eN was eluted by increasing concentration of NaCl from each column and was detected by Western blot.
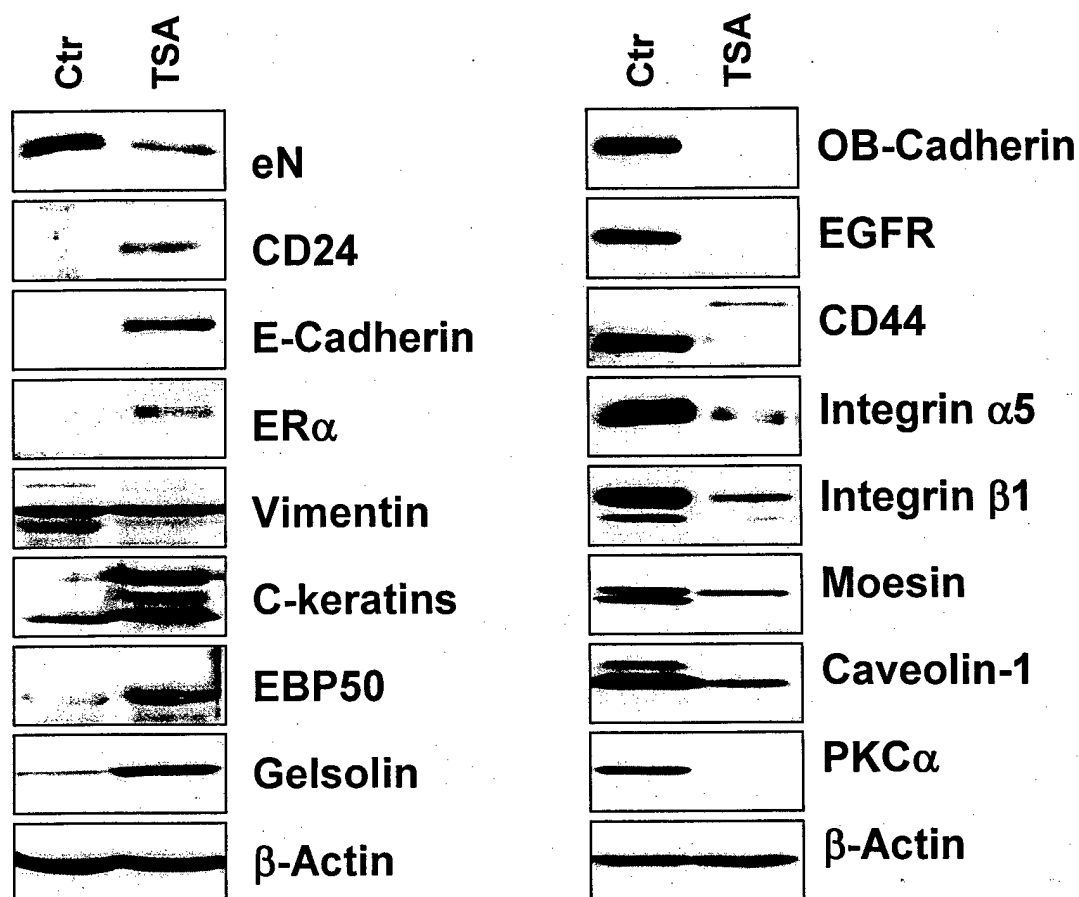
Fig. 5. The effect of TSA (48 hours at 0.5 uM) on the expression of several membrane and signaling proteins in MDA-MB-231 cells.
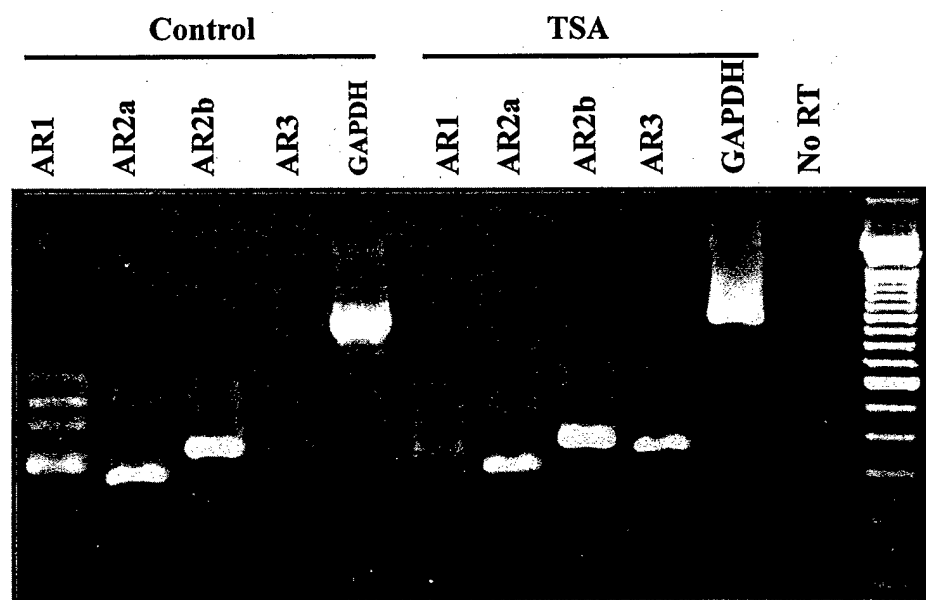

Fig. 6. The effect of TSA (24 hours at 1 uM) on the expression of adenosine receptors mRNA in MDA-MB-231 cells determined by RT-PCR.

suggest that such anti-metastatic activity, at least in the subset of ER(-) breast cancer cells, may be due to the reversion of intrinsically invasive and metastatic mesenchymal phenotype. More details on this subject in Appendices D.

3. Key Research Accomplishments

- We have defined mesenchymal cell phenotype typical for cell expressing high eN in advanced breast carcinoma. This highly inasive cell phenotype is characterized by high rate of extracellular adenossine generation. We have also determined that HDAC inhibitor TSA reverses the mesenchymal phenotype and leads to decresed expression of eN. Manuscript on this topic has been submitted to Molecular Cancer Therapeutics (2004) (Appendices D). In addition, in the course of this work, a review on 5'-nucleotidases has been written and accepted in J. Biol. Chem. (Appendices E)
- We have discovered that eN is a specific receptor for Tenascin C and characterized the relationship between these two proteins in terms of adhesion, generation of adenosine and cell migration.
- In initial inoculations of nude mice with eN_{low}, ADA and GFP MDA-MB-231 cells we found that grafts expressing low eN grew slower than GFP and ADA expressing cells. The growth rate of xenografts depends on the number of cells inoculated.

4. Reportable Outcomes

Our in-depth characterization of w/t and modified breast cabcer MDA-MB-231 cells revealed that the high potential to generate adenosine is typical for mesenchymal cells. The characteristic feature of these cells is that they are more motile and invasive and in breast cancer are more common for the advanced stage of the disease. This clear link between aggressive cell behavior and breast cancer progression on one hand, and the capacity to generate adenosine on the other hand, further supports for the role of eN and adenosine in breast cancer. The specific interaction between eN and Tenascin C is a novel observation and may provide a basis for the mechanism of specific interactions between invasive cells and the substratum at the invasive end of breast carcinoma. We are also proposing the potential mechanism of HDAC inhibitors in breast cancer treatment: the reversion of mesenchymal phenotype that may be defined as Mesenchymal to Epithelial Transdifferentiation (MET). Finally, preliminary testing showed that cells expressing low eN have lower tumorigenic potential in nude mice than control cells. Further testing is necessary to define the role of adenosine and eN in the mechanism of growth retardation of tumor grafts.

5. Conclusions

During 3 year period we have accomplished several goals set for this project. We have performed in-depth characterization of genetically modified cell lines and tested them in preliminary inoculations. The results obtained so far clearly support our initial hypothesis on the role of adenosine in breast cancer progression. As an additional bonus, the discovery of interactions of eN with Tenascin C, will strengthen the proposed novel functions of eN and adenosine in breast cancer. The effect of HDAC However, we were unable to obtain definitive results on xenograft growth in nude mice. Given the importance of this project, and a strong preliminary evidence on the role of eN and/or adenosine in breast cancer progression, we are continuing this project using other funding means. Currently, we have initiated a new set of inoculations of MDA-MB-231 cell derivatives to test their growth rate and angiogenic potential in nude mice. These results are being used as a basis for new grant application in NIH.

Personnel involved in this project:

Jozef Spychala, Ph.D. P.I.
Anna Ostapkowicz, Tech II
Rafal Sadej, Tech II
Elzbieta Kulig, Post-Doc

6. References

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7. Appendices

5 Items:

A. Abstract from the 9th Purine and Pyrimidine Metabolism in Man Meeting in Amsterdam June 9th-13 2003.

B. Abstract from Biochemical Society Meeting in Gdansk, September 5th-8, 2003

C. Abstract from Purinergic Meeting in Chapel Hill, June 5th-7, 2004

D. Lipid rafts remodeling Lipid rafts remodeling in ER negative breast cancer is reversed by histone deacetylase inhibitor." by Ostapkowicz Anna, Inai Kunihiro, Smith Leia, Kreda Silvia and Spychala Jozef, submitted to Molecular Cancer Therapeutics (2004).

E. Publication entitled "Mammalian 5'-nucleotidases" by Vera Bianchi and Jozef Spychala., J. Biol. Chem. 278, 46195-46198, 2003

Appendices A

The association of ecto-5'-nucleotidase (eN), integrin β1, EGFR and vimentin with invasive breast carcinoma: The role of eN in invasive phenotype.

Spychala Jozef, Ostapkowicz Anna, Naiki Hironobu, Noriki Sakon, Fukuda Masaru and Inai Kunihiro

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Breast carcinoma is a heterogeneous disease and expression profiling has identified several subtypes that may be useful in determining more effective individualized treatment regimens in the clinic. Previously we have found ecto-5'-nucleotidase overexpression in ER negative breast cancer. In order to associate the expression of eN with specific cell phenotype, we have performed expression profiling at the level of protein in several breast cancer cell lines. Our focused analysis of membrane and cytoskeletal proteins revealed that eN is specifically co-expressed with a number of membrane proteins, such as EGFR, CD44, N-cadherin, OB-cadherin, caveolin, integrin β1 and integrin α5. Among cytoskeletal proteins eN co-expresses with vimentin, merlin, fascin, moesin and spectrin. Also several signaling molecules, such as tyrosine kinase Lyn, trimeric Gαi and PKC tightly co-express with eN. This expression profile is characteristic for normal fibroblasts and was also found in more aggressive and tumorigenic breast cancer cell lines that have undergone Epithelial to Mesenchymal Transdifferentiation (EMT). Since eN and several indentified membrane proteins, that either co-express or have exclusive expression pattern, are components of lipid rafts, these results suggests that there is a significant remodeling of this membrane microdomain in EMT. The co-expression of eN with vimentin also correlated with the responsiveness to ConcanavalinA in more aggressive breast cancer cell lines suggesting functional association. Preliminary survey of clinical samples from breast cancer patients show coexpression of eN with Integrin β1 and vimentin at the invasive edge of the tumor. Thus, these data suggest that elevated expression of eN and increased potential to generate extracellular adenosine may have specific functions related to cell migration and strengthen the significance of this protein as a novel marker for invasive and metastatic breast carcinoma.

Appendices B

Regulation and function of ecto-5'-nucleotidase and adenosine in cancer

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Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, USA and Fukui Medical University, Fukui, Japan

Adenosine has potent angiogenic, cytoprotective and anti-inflammatory functions that have been documented in several tissues. Increasing evidence suggests that similar functions of adenosine may contribute to solid tumor progression. Since in epithelial cells adenosine is generated by ecto-5'-nucleotidase (eN), we focused our analysis on the expression and regulation of eN in cancer. We found that eN is upregulated by several oncogenes, including c-jun, β–catenin and wnt. In breast carcinoma eN expression is suppressed by ERα and loss of estrogen receptor is prerequisite for high eN expression. Expression profiling of breast cancer cells revealed that eN expression is tightly associated with metastatic phenotype that is more typical for ER(-) cells. Among markers characteristic for invasive cancer cells eN co-expresses with CD44, EGFR, MDR1, Integrins α5β1, and α3β1 and vimentin. Other cell surface antigens previously established as markers of breast crcinoma, such as CD24 and uPAR, were expressed in cells that lack eN. Remarkably, the expression profile of membrane, cytoskeletal and signaling molecules is almost identical in WI38 fibroblast suggesting that transition to ER(-) phenotype follows epithelial to mesenchymal transdifferentiation (EMT). Analysis of eN expression in clinical breast cancer samples confirmed negative correlation with ER receptor status. In addition, preliminary assesment of eN expression in ER(-) breast cancer samples showed high eN expression at the invasive end of cancer lesions and co-expression with vimentin and integrin β1. The increased expression of eN in cancer cells was also associated with altered adhesive and migratory properties and we hypothesize that either higher level of produced adenosine by eN or interaction of this membrane protein with the ECM and modulation of integrin function may contribute to more aggressive behaviour of ER(-) and metastatic cancer cells. Supported by DOD Grant DAMD17-01-1-0351.

Appendices C

Defining the interactions of Ecto-5'-nucleotidase and Tenascin C and the effects on adenosine generation in cancer cells.

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Tenascin C is expressed at elevated levels in invasive human solid tumors; however its specific role in cancer biology remains obscure. Previously, we have found that ecto-5'-nucleotidase (eN) is a marker of ER (-) breast carcinoma and elevated expression correlates with more invasive mesenchymal cell phenotype. To test for the potential interactions between eN and tenascin C we measured adenosine generation from AMP in cells treated with soluble components of extracellular matrix (ECM) and found that, among several ECM components tested (collagen, fibronectin, laminin, vitronectin and restrictin R), tenascin C was the only ECM component that strongly inhibited *in situ* ecto-5'-nucleotidase (eN) activity and adenosine generation from AMP in breast cancer cells. The inhibition was comparable to that induced by concanavalin A, a strong and well-defined inhibitor of eN. To investigate for the potential role of eN in cancer cell adhesion and motility, we tested cell adhesion in cells plated on different ECM. MDA-MB-231 breast cancer cells expressing high level of eN, in contrast to cells with expression downregulated by antisense eN cDNA, were attached better on tenascin C than on other extracellular matrices. Furthermore, breast cancer cells with 90-95% suppressed expression of eN migrated 20 - 30 % faster on tenascin C than wild-type cells. These data suggest that eN may be a novel and specific receptor for tenascin C and that the interaction between these proteins may alter cell adhesion and lead to decreased local generation of adenosine. In light of known functions of adenosine in cell adhesion, our findings suggest that eN is a novel ECM-sensing factor with potential functions in cancer cell invasion. Supported by DOD Grant DAMD17-01-1-0351.

Lipid rafts remodeling in ER negative breast cancer is reversed by histone deacetylase inhibitor[1]

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Appendices D

Abstract

Expression profiling of breast cancer cell lines and clinical samples revealed a significant differences in gene expression in ER negative breast cancer. Recently, we have found dramatic overexpression of eN (ecto-5'-nucleotidase or CD73), a GPI-anchored component of lipid rafts, in ER(-) breast cancer cell lines and in clinical samples. To find out whether there is a more general shift in expression profile, we undertook an investigation on the expression of specific group of membrane and cytoskeletal proteins that associate with lipid rafts in more aggressive and metastatic breast cancer cells. Our analysis revealed a remarkably uniform shift in expression of a broad range of membrane, cytoskeletal and signaling proteins in ER(-) cells. A similar change in expression profile was found in two *in vitro* models of transition to ER-negative breast cancer: drug resistant Adr2 and c-Jun transformed clones of MCF-7 cells. These results suggest that there is a major remodeling of lipid rafts and underlying cytoskeletal network during transition to ER negative breast cancer. Similar expression pattern was observed in normal fibroblasts, suporting the emerging view of the commonality of membrane determinants of invasive cancer cells with normal mesenchymal phenotype. Interestingly, this phenotype was associated with increased capacity to generate extracellular adenosine and signaling through A1 adenosine receptors. To test whether the mesenchymal phenotype could be reversed by treatment with differentiating agent, we treated ER(-) negative cells with TSA, an inhibitor of histone deacetylase, and observed broad reversal of mesenchymal and re-appearance of epithelial markers. Thus, our results suggest that during transition to more invasive breast cancer there is a significant structural re-organization of lipid rafts and underlying cytoskeleton that is reversed upon histone deacetylase inhibition.

Appendices D

Introduction

Establishment and clinical use of tumor markers that define invasive and metastatic breast carcinoma is critical for more individualized therapeutic strategies in the future. Since breast cancer is becoming an increasingly heterogeneous disease, the task of defining specific cancer cell phenotypes is especially challenging. Several individual breast cancer markers have been established and proven useful for target-specific pharmacological intervention. The clinically proven therapies include targeting estrogen and progesteron receptor and more recently Erb2 and EGFR. Major differences in expression profiles of wide number of genes has been documented in ER(-) and ER(+) carcinomas (1), and both in *in vitro* studies and in the clinic, these differences were associated with either more motile and invasive phenotype or more aggressive course of the disease in the case of ER(-) breast carcinoma (2). Membrane proteins and associated cytoskeleton mediate the communication with the extracellular milieu and the composition of membrane proteins is critical for cell behavior in general and invasive and metastatic properties in particular.

Among different membrane microdomains, lipid rafts are one of the least understood subcellular elements. Although their lipid composition has been investigated in several studies, (3-7), protein components were not systematically compared between invasive and noninvasive cells. Since several *in vitro* models of breast cancer exemplify transition to more invasive and metastatic state, we have chosen this model to study changes in lipid rafts composition after loss of ER expression. Our recent finding that ER(-) breast cancer cells express high level of eN (ecto-5'-nucleotidase), a GPI-anchored protein and a marker of lipid rafts, provides an early argument for the lipid raft remodeling during transition to more aggressive breast carcinoma (8). In this study, we aimed to analyze whether there is a consistent alteration in expression of

Appendices D

cytoskeletal membrane and lipid raft protein components across a wider population of ER

positive and negative cells that would suggest a coordinate expression consistent with the motile

and invasive phenotype. Although limited by the availability of suitable antibodies, the focus on

expressed proteins rather than mRNA allowed us to directly relate protein expression with the

specific cell phenotype.

Materials and Methods

Cell lines. Breast cancer ER positive cells lines MDA-MB-474, ZR-75-1, MCF-7 and

negative SK-BR-3, MDA-MB-468, MDA-MB-435s, MDA-MB-231, BT-549, Hs578t,

nontrasformed MCF-10A, c-Jun transformed MCF-7/c-Jun clone 2-33 and control MCF-7/neo

clone 7-1 and human fibroblasts WI-38 were obtained from either Tissue Culture Facility at

LCCC/UNC, ATCC or developed as described before (9). The Adr2 and AdrR MCF-7

adriamycin-resistant cell sublines was from Dr Y.M. Rustum (RPCI, Buffalo, NY). Cells were

maintained in MEM supplemented with Eagle salts, NaPyr, nonessential amino acids and 10%

FBS (most cell lines), in McCoy's supplemented with 15% FBS (SK-BR-3 cells), in Leibowitz

L-15 supplemented with 10% FBS (MDA-MB-468 cells) and in MEGM medium supplemented

with BPE, hEGF, Insulin, Hydrocortisone and 10% FBS (BioWhittaker medium for MCF-10A

cells) in CO_2/O_2 atmosphere at 37° C, except for MDA-MB-468 cells which were grown at

ambient atmosphere at 37° C. All media contained penicillin and streptomycin.

Reagents. All general reagents were ACS or the highest purity commercially available.

The following antibodies were used. Rabbit polyclonal anti-eN antibodies (for Western) were

generated as described before (10) or purchased from BD Biosciences (PharMingen 5502 56)

(for IF). Anti-Gαs/olf sc-383, Gαi-2 sc-7276, Gα12 (sc-409), Gα13 (sc-410), Thy-1 sc-9163,

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CD24 sc-11406, Gβ sc-378, Gβ2 sc-380, ankyrin B (clone 2.20), cyclin D1 (sc-8396), Integrin β1 sc-8978, Integrin β2 sc-6624, Integrin β3 sc-6627, ERα sc-8005, fyn sc-434, lyn sc-15, lck sc-433, c-fgr sc-130, hck sc-72, MDR1 sc-8313, N-cadherin sc-8424, OB-cadherin sc-9997, caveolin-1 sc-894, PKCα sc 8393, Fascin, sc-16579, lamin B1, sc-6216 and secondary HRP conjugated against goat and rat IgG were from Santa Cruz (Santa Cruz, CA). E-cadherin C20820, FAK F15020, integrin β1 I41720, integrin α5 I55220, PKCβ P17720, PKCδ P36520, PKCθ P15120, moesin M36820, EBP50 E83020, ezrin M36820, gelsolin G37820, flotillin-1 F65020 antibodies were from Transduction Laboratories (Lexington, KY). Anti-CD44s 13-5500 antibody was from Zymed (San Francisco, CA). Anti-c-Yes antibody 06-514 and c-Src GD11 were from Upstate (Lake Placid, NY), rabbit anti-uPAR (399R) were from American Diagnostica Inc. (Greenwich, CT), anti-β-actin were from Oncogene (Boston, MA), anti-filamin antibody MAB1678, integrin αv AB1930, talin MAB1676, vimentin AB1620 were from Chemicon, Temecula, CA, and anti-intestinal alkaline phosphatase antibodies were from Biogenesis, Kingston, NH. Antibodies against cytoskeletal proteins α-smooth muscle actin (clone 1A4), cytokeratin (clone K8.13, detecting forms 1, 5, 6, 7, 8, 10, 11, 18 and clone K8.12, detecting forms 13, 15, 16) were from Sigma, St Louis, MO.

Lipid rafts isolation. Cell lysates were prepared by mixing equal volumes of cell pellets with 2 % Triton X-100 on ice for 1 minutes and subsequent dilution 2x with PBS and further 2x with 35% Nycodenz in PBS. At each step mixing was achieved by pipetting the lysate up and down several times with eppendorf pipettor. A modified procedure for density gradient centrifugation using Nycodenz {5'-(N-2,3-Dihydroxypropylacetamido)-2,4,6-Triiodo-N,N-bis(2,3-Dihydroxypropyl) -isophtalamide}from Sigma-Aldrich (St. Louis, MO) was used to fractionate Triton X-100 soluble and insoluble membrane and cytoskeletal subdomains and

complexes [Hostager, 2000 #12454]. For the purpose of centrifugation, cell lysates containing 3 to 4 mg of total protein were diluted 2-fold with 35% nycodenz. Density step-gradient was generated by applying 0.5 ml aliquots of increasing concentration of Nycodenz (35%, 25%, 22.5%, 20%, lysate in 17.5%, 15%, 12%, 8% and 4%) sequentially into Beckman (Palo Alto, CA) 13 x 51 mm polyallomer tubes. Note that lysates were placed in the middle of Nycodenz gradient (Figure 1A). Tubes were centrifuged at 46K for 4 hrs in a Beckman 55Ti rotor at 4°C. Following centrifugation 0.5 ml fractions were carefully withdrawn and small pellet was resuspended in PBS containing 0.5% SDS and 1% Triton X-100 (fraction 10). Total of 10 fractions and control input lysate were analyzed for the distribution of proteins by Western blot. Typically, components of light lipid rafts and caveolae distributed into first 4 fractions, soluble cell components including cytosolic proteins remained in fractions 5 and 6 and cytoskeleton-associated high density fractions were distributed in fractions 7 to 9.

Western blotting. Cell extracts, obtained by scraping cells in PBS in the presence of protein phosphatase and protease inhibitors and lysing with 1% Triton X-100/PBS were loaded on the SDS-PAGE at 30 ug per lane. Separated proteins were transferred onto Immobilon-P 0.45 μM (Millipore, Bedford MA) PVDF membrane and used for probing with specific antibodies. Two buffer systems were used during incubations with antibodies: PBS supplemented with 5% Carnation fat-free dry milk and 0.2% Tween 20 or 25 mM Tris, pH 8.4 supplemented with 130 mM NaCl, 5 mM potassium phosphate, 5% fat-free dry milk and 0.2% Tween 20. Blots were re-used several times after mild stripping by air-drying o/n at RT when necessary. Secondary antibodies conjugated to HRP and BM Chemiluminescence Western Blotting Kit (Roche, Indianapolis IN) were used to develop images on Kodak (Rochester, NY) X-Mat Blue XB-1 film.

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Cholesterol assay. The cholesterol content in density gradient fractions was assayed using Sigma Corp. colorimetric assay.

RT-PCR. Extraction of total RNA was performed using TRI-reagent from MRC (Cincinnati, OH).

Results

The modified procedure of density gradient centrifugation using Nycodenz was employed and tested using several marker proteins that represent lipid rafts, cytoskeleton and soluble cell compartments (Figure 1). As shown in Figure 1B eN, a marker of lipid rafts was found in predominantly in fractions 1 to 4, which represent a low density lipid raft fraction of cell lysate. On the other hand, α-tubulin (dis-assambled to monomers under low temperature conditions) and adenosine kinase (AK) were distributed predominantly to fractions 5 to 7, which represent Triton X-100 soluble cell extracts. In contrast, vimentin, β-actin and lamin B1 were distributed mostly to fractions 5 to 10 which represent both Triton X-100 soluble and insoluble fractions of cell lysates. Interestingly, small portion of vimentin was also found in low density lipid raft fractions suggesting that this protein may directly interact with components of lipid rafts. Flotillin, another marker of lipid-rich low density fractions distributed exclusively to low density fractions 3 and 4. However, thy-1 – another GPI-linked protein known to associate with lipid rafts, was found exclusively in high density cytoskeletal fractions. This finding, and some presence of eN in the same cytoskeletal fractions 7 to 9, suggests that these high density fractions represent lipid rafts associated (anchored) with cytokeletal elements. To verify this possibility we have analyzed density gradient fractions for the presence of cholesterol, a lipid raft chemical component. Figure 1C shows that cholesterol distributed into two well defined peaks: major peak in fractions 1 to 4

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and a minor peak in fractions 7 and 8. Thus, these findings suggest that thy-1-containing lipid rafts constitute a distinct raft subtype that is constitutively associated with cytoskeletal elements in MDA-MB-231 cells.

We further used the MDA-MB-231 breast cancer cell lysates to determine the cellular distribution of several membrane and cytosolic signaling proteins that may, at least temporarily, associate with lipid rafts. Figure 2A shows that src-family members, with the exception of yes, distributed mostly to lipid rafts. In contrast, protein kinase Cα was found exclusively in soluble cell compartment (Figure 2B). CD44 and Gαi2, a known lipid raft components also distributed mostly to lipid rafts. On the other hand Gαs and Gβ2 distributed in significant portion to soluble and cytoskeletal compartments (Figure 2B). Integrin β1, EGFR and OB-Cadherin – proteins highly expressed in MDA-MB-231 cells, were distributed in all three cell compartments and FAK was found only in soluble and cytoskeletal fractions. Analysis of cell lysates from ER-positive breast cancer MCF-7 cells, that express very low levels of eN, also showed lipid rafts distribution of this protein, along with GPI-linked CD24, src, lyn and Gαs (Figure 2C). These results may suggest that lipid rafts components distribute to specific compartments independently of cell type and raise the possibility that protein composition of lipid rafts may differentiate cells of different phenotypes.

To address this possibility we performed a limited expression profiling of a number of membrane and lipid rafts-associated signaling molecules in breast cancer cells that represent ER(+) and ER(-) phenotypes. We used a broad panel of cell lines, which invasive and metastatic potential have been defined in previous studies (11), thus enabling us to correlate protein expression with specific cell phenotypes. In this cell panel MCF-10A cells serve as non-tumorigenic mammary epithelial control and SK-Br-3 and MDA-MB-468 represent ER(-) cells

that are less tumorigenic than MDA-MB-435s, MDA-MB-231, BT-549 and Hs578t cells (11).

Previously we have reported dramatic upregulation of eN, a GPI-anchored membrane ecto-

protein, in ER(-) breast cancer cells (12). Here we compared the expression of eN with other

lipid raft components such as CD24 and alkaline phosphatase. As shown in Figure 3A, the

expression of the proposed breast cancer marker CD24 concurred with the ER receptor status,

and was also found in SK-Br-3 and MDA-MB-468 cells. A reciprocal pattern was found for

intestinal alkaline phosphatase and eN. This uniform expression profile prompted further

investigation of other membrane and cytoskeletal proteins, especially those that may associate

with lipid rafts. As shown in Figure 3 A-F, the broader protein survey revealed a striking

alteration in expression of specific proteins in ER(+) and ER(-) cell lines with somewhat variable

expression in SK-Br-3 and MDA-MB-468 cells (Figure 3 A-F). While E-cadherin was expressed

mostly in ER(+) and control cells, other adhesion receptors integrins $\beta1$, $\alpha5$ and αV, CD44 and

OB and N cadherins tended to co-express with eN in ER(-) cells (Fig. 2 A-B). Similarly,

cytoskelatal protein vimentin and partially smooth muscle actin, in contrast to several

cytokeratins, coexpressed with eN in ER(-) cells. Lamin B1 expression was independent of cell

type, however, α-tubulin tended to express at higher levels in more aggressive ER(-) cells

(Figure 3 C). Other membrane or membrane-associated signaling molecules such as EGFR, Lyn,

PKCα also showed similar expression pattern (Figure 2D). Analysis of proteins associated with

cytoskeleton show that while EBP50 and gelsolin were associated with less invasive cells,

fimbrin, talin, filamin and especially fascin and moesin tended to express at higher level in more

invasive breast cancer cells (Fig. 2D and E). Interestingly, caveolin-1 expression strongly

coincided with eN further suggesting that, in addition to eN lipid rafts, caveolae may have

specific function in more invasive cells.

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Based on this comprehensive expression analysis we may subcategorize breast cancer cells into three distinct profiles: BT474, ZR-75-1 and MCF-7 cells having epithelial and MDA-MB-435s, MDA-MB-231, BT-549 and Hs578t having mesenchymal features. Interestingly, cell lines SK-Br-3 and MDA-MB-468 fall in-between: while losing many epithelial markers such as E-cadherin and certain cytokeratins, they did not yet acquire full set of mesenchymal features. During long cell culture we occasionally observed temporal expression of eN in SK-Br and MDA-MB-468 cells suggesting some intrinsic phenotypic plasticity in these particular cells. This could explain metastatic behavior of MDA-MB-468 cells in mouse xenograft model (13).

To further test whether *in vitro* transition to ER(-) status and more invasive phenotype will show similar shift in expression profile, we used two independent *in vitro* models of breast cancer progression. Development of drug resistance and overexpression of c-Jun in MCF-7 cells were both shown to correlate with loss of ER expression and transition to more invasive and tumorigenic phenotype (9, 14). In these cells we focused our analysis on those membrane, lipid raft and cytoskeletal proteins that showed differential expression in a broader panel of breast cancer cells (Figures 1 to 3). Results presented in Figures 4A and 4B demonstrate that in these two models there is a very similar shift in expression profile to that in ER(-) cells presented in Figures 1 to 3. To further analyse whether membrane or cytoskeletal protein profiles may differentiate between "cancer metastatic" and normal motile phenotypes, we included in our analysis normal human fibroblasts WI-38. Data presented in Fig. 4A and 4B show that drug resistant, c-Jun-transformed and all other invasive cells shown in Figures 1 to 3 exhibit the expression pattern of membrane and cytoskeletal proteins that is remarkably similar to normal fibroblasts. In addition to strongly increased expression of lyn, data showed in Figure 4B also

show upregulation of Src and downregulation of Lck in ER(-) cells and in fibroblasts. These observations were confirmed using a broader panel of breast cancer cell lines (data not shown).

Previous studies demonstrated that histone deacetylase inhibitors caused shift in expression pattern of selective protein groups. In breast cancer cells, TSA has been shown to induce the expression of ERα and E-cadherin in MDA-MB-231 cells. Based on these data we asked whether TSA may reverse the expression of broader range of mesenchymal proteins that define the invasive and metastatic phenotype, as shown in Figs 1 to 3. Treatment of MDA-MB-231 cells with 0.5 μM TSA for 48 hours caused downregulation of eN protein (Figure 5). Similar result was seen with eN mRNA (not shown). Other proteins which have been shown to contribute to invasive cell behavior, such as CD44 (standard form), PKCα, caveolin-1, integrins β1 and α5, EGFR, OB-cadherin, moesin, were all strongly downregulated by TSA. In the case of CD44 a larger form of the protein, previously seen in MDA-MB-468 cell line (Figure 3 A) was expressed instead of CD44s. On the other hand, epithelial markers such as cytokeratins (detectable with K8-13 antibody), E-cadherin, ERα, EBP50 and gelsolin were upregulated. The expression of CD24, another marker of lipid rafts in ER(+) cells, was also upregulated. The expression of several other proteins, including Lyn (not shown) and vimentin was only slightly downregulated, however, at 48 hours of drug treatment, there was increased incidence of apoptosis that may have masked further changes in protein expression. Along with the complete disappearance of CD44 standard form we have noticed an appearance of a higher molecular form, probably a splice variant, that was also seen in untreated MDA-MB-468 cells (Figure 3 A). Adenosine producing eN was dramatically increased in mesenchymal cells (Fig. 3A and 4A) and was decreased upon TSA treatment (Fig. 5A). Furthermore, the expression of adenosine receptors were unevenly affected by TSA: whereas A2a and A2b were unchanged, the

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expression of A1 was decreased and A3 was increased during transition to epithelial phenotype (Fig. 5B). Thus, increased capacity to produce adenosine correlates with increased signaling through A1 receptors in aggressive breast cancer cells and is reversed by histone deacetylase inhibitors. We conclude that in breast cancer model TSA induced changes in expression pattern of membrane and cytoskeletal proteins that reverse changes seen during progression to ER(-) breast cancer phenotype and lead to re-establishment of epithelial markers. Overall these changes seem to parallel cell differentiation and in the context of mesenchymal cells may represent mesenchymal to epithelial transdiferentiation (MET), a process reciprocal to EMT.

Discussion

The analysis of expression of membrane, cytoskeletal and associated proteins in 12 breast cancer cell lines show that there is consistent shift in expression pattern between non-invasive and invasive cell phenotypes. Our focus on specific set of known membrane and cytoskeletal proteins allows us to directly correlate the expression pattern with well-defined specific cellular phenotypes. Analysis of lipid components of membranes in breast cancer cells was performed previously and revealed significantly altered ratio of phospholipids (3), increased level of gangliosides, components of lipid rafts (4-6) in ER(-) cells. Importantly, increased circulating levels of gangliosides were found in breast cancer patients when compared with healthy individuals (7). Although association of few membrane and cytoskeletal proteins with the ER status were reported before, no comprehensive analysis of membrane and cytoskeletal proteins in a broad cell panel of breast cancer cells has been performed thus far. Among these proteins vimentin, EGFR, CD44, fascin, E-cadherin were highly correlated with ER status both in breast cancer cell lines and in clinical samples (15). Our survey has significantly extended this

correlation and established a broader set of membrane, lipid raft and cytoskeletal proteins that define the motile and invasive phenotype. Remarkably, the expression profile, common to all 6 more aggressive cell lines (MDA-MB-435s, MDA-MB-231, BT549, Hs578t, MCF-7/Adr2 and MCF-7/c-Jun), was to large extent recapitulated in normal fibroblasts. The extent of similarities, including increased expression of signaling molecules such as EGFR, PKCα, Lyn, Gαi-2, suggests that both structural proteins and regulatory circuitry has been adopted from normal mesenchymal cells. Thus, our results support the occurrence of epithelial to mesenchymal transdifferentiation (EMT) in breast cancer (16, 17).

A clearly distinct expression profile has been observed in MCF-10A cells that, despite spindle cell morphology, are frequently used as model for nontransformed breast epithelial cells. High expression of smooth muscle actin and a co-expression of cytokeratins and vimentin suggest that this cell line is potentially of myoepithelial/basal cell origin, and thus represent a distinct subset of cells that may originate from mammary gland epithelium.

We have found that several membrane proteins residing in lipids rafts are differentially expressed in invasive breast cancer cells, suggesting that there is a major remodeling of this membrane microdomain during breast cancer progression. GPI-linked proteins are typically considered markers of lipid rafts and in our study they show most dramatic shift in expression profile. The complete downregulation of CD24 in ER(-) cells and the emergence of eN and ALP most likely have physiological consequences. CD24 is a new marker for breast and ovarian carcinoma [Fogel, 1999 #9649; Kristiansen, 2002 #12472] with positive estrogen receptor status (18). This mucin-like heavily glycosylated protein was shown to be a ligand for P-selectin and proposed to mediate rolling in endothelium (19). On the other hand, eN and ALP that seem to replace CD24 during breast cancer progression, both have phosphohydrolase activity and

participate in dephosphorylation of extracellular nucleotides (mostly adenosine phosphates) and generation of signaling adenosine (20). Adenosine, the product of both eN and ALP enzymatic activities (21), acting through a family of specific receptors, has well established roles in adhesion, growth regulation, vasodilation and angiogenesis that may be important for breast cancer progression (reviewed in (20, 22)). Interestingly, TSA treatment in MDA-MB-231 cells decreases the expression of A1 and increases the expression of A3 adenosine receptors, suggesting that there is a shift in signaling properties of adenosine in invasive and metastatic cells. Wnt-1 signaling pathway that has important role in initiating EMT has been shown recently to upregulate eN expression (23). eN has been also reported to participate in cell adhesion (24, 25), however, it is at present unclear how eN would specifically contribute to invasive cell behavior.

In addition to more obvious changes in expression of cadherins, cytokeratins and vimentin during progression to metastatic breast carcinoma, there are more subtle, but nevertheless consistent, changes in expression of underlying cytoskeleton-associated proteins. The down-regulation of EBP50 and gelsolin, two proteins independently associating with actin cytoskeleton, correlate with upregulation of moesin, fascin and to lesser extent talin and filamin. EBP50 may associate with ERM proteins (Ezrin, Radixin, Moesin) and thereby regulate anchoring of lipid rafts to cytoskeleton [Itoh, 2002 #12475]. A number of functional associations between proteins that overexpress in mesenchymal cells have been established. CD44/moesin and CD44/Lyn interactions collaborate in triggering invasiveness and chemoresistance (26, 27). Vimentin and fimbrin form functional complexes in macrophages (28). Thus, all these proteins seem to have distinct roles in aiding the assembly of structural links between actin or vimentin cytoskeleton and membrane microdomains. In that capacity, they are well suited to regulate

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adhesion/deadhesion and membrane fluidity that is critical for efficient cell motility. Although it is well accepted that metastasis requires cells to become motile and invasive, the molecular basis of cell migration, and underlying genetic changes that induce the motility, are not well defined. Our analysis identified a broad group of membrane, lipid raft and cytoskeletal proteins that appear to change as a "package" representing either epithelial or mesenchymal phenotypes. Although many other proteins will be likely identified, those defined in this work may be a starting point for the use in functional *in vitro* studies and, as a markers, in the clinic.

It is not yet clear whether expression profile exemplified here by ER(-) cells *in vitro* represents a distinct clinical subset of advanced breast carcinoma. A number of membrane and cytoskeleton-associated proteins shown to be elevated in this survey were also shown to co-express in clinical setting, thus suggesting that specific protein groups may functionally co-express during cancer progression *in vivo*. We have shown recently that eN is expressed in ER(-) breast carcinoma. EGFR and vimentin were shown to co-express in specific subset of advanced breast carcinoma distinct from both erbB2-positive and ER-positive cases (15). CD44, moesin, and fascin were also found to express at higher level in advanced breast cancer (29-31). Recent study found a striking correlation of CD44-positive and CD24-negative cells derived from breast carcinoma with their tumorigenic potential (32). Although vast majority of normal mammary epithelial cells are ER(-) most human breast carcinomas are ER(+). Altered ER status during breast cancer progression has been proposed to explain lost responsiveness to hormonal therapy and more aggressive course of the disease. However, there seem to be a controversy whether loss of ER expression is a frequent clinical stage during disease progression. The transition to ER(-) has been recapitulated *in vitro* with ER(+) breast cancer cell lines (9) and was associated with the development of drug-resistant, invasive and metastatic phenotype. In MCF-10A cells, that are

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frequently used as a model of pre-malignant phenotype, a spontaneous shift between ER negativity and positivity has been reported in long term culture (33) and a derivative MCF-10TA generated heterogeneous malignant variants that were both ER(+) and ER(-) (34), suggesting that such shift is possible. Furthermore, an MDA-MB-231 metastatic breast cancer cell line treated with histone deacetylation inhibitor trichostatin A re-expresses estrogen receptor and E-cadherin, proteins typical for more differentiated ER-positive breast carcinoma (35). A strong argument for the transition from ER positivity to negativity was obtained in animal model of breast cancer where the large T-antigen and polyoma middle T-antigen transgenic mouse mammary tumor progression was accompanied by the loss or decrease of ERα expression (36-38).. However, it is still unclear whether decrease in ER positivity after anti-estrogen receptor therapy in humans represents a transition to ER(-) carcinoma or is just a temporary decrease in ER expression and a definitive evidence for such transition in the clinic is still lacking (39).

Inhibitors of histone deacetylases (HDAC), including TSA, have potent effects on global gene expression, inhibit cell proliferation and induce differentiation and apoptosis (40-42). Although HDAC inhibitors caused changes in many different cell types, mesenchymal cells were proposed to be particularly sensitive (43). In particular, previous studies have shown that MDA-MB-231 cells induced the expression of E-cadherin and ERα after treatment with HDAC inhibitor TSA (35). Given the known genome-wide effects of HDAC inhibitors, such response suggested that TSA may have induced differentiation, which in the context of mesenchymal cells may be regarded as mesenchymal to epithelial transdifferentiation (MET) (44). Indeed, the expression of most markers that differentiate ER(-) and ER(+) cells were affected in a reciprocal manner. Thus, our expression profiling of a broader set of membrane and cytoskeletal proteins provide a strong evidence for the MET after treatment of breast cancer cells of mesenchymal

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phenotype with TSA. Previous studies have shown that histone deacetylase inhibitors increased the expression of metastasis suppressors such as breast metastasis suppressor 1 (BRMS1) (45), tissue inhibitor of metalloproteinase 3 (TIMP3) (46) and nm23 (47) and thereby may have therapeutic activity in metastatic phase of breast and other carcinomas. In this context our results suggest that such anti-metastatic activity, at least in the subset of ER(-) breast cancer cells, may be due to the reversion of intrinsically invasive and metastatic mesenchymal phenotype.

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Appendices D

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Appendices D

Legends to Figures

Fig. 1.

A) Scheme illustrating Nycodenz density gradient centrifugation. B) Distribution of several cytskeletal, membrane and soluble proteins from MDA-MB0231 cells after density gradient centrifugation. C) Distribution of cholesterol after density gradient centrifugation. Twenty ul from each fraction collected after centrifugation, and 30 ug protein from cell lysate, were loaded onto each lane and separated on SDS-PAGE as described in Materials and Methods. Each experiment was repeated, with similar outcome, at least twice.

Fig. 2.

Distribution of proteins from MDA-MB-231 and MCF-7 cells after density gradient centrifugation. A) and B) Proteins from MDA-MB-231 cells associated with lipid rafts. C) Proteins from MCF-7 cells associated with lipid rafts. Note that eN expression in MCF-7 cell fractions was visualized only after 20 times longer exposure of blots. Other conditions as in Fig 1.

Fig. 3.

Differential expression of membrane and cytoskeletal proteins in hyperplastic MCF-10A cells and a panel of breast cancer cell lines. A) Representative lipid rafts associated proteins, B) Representative membrane proteins with role in adhesion. C) Cytoskeletal proteins, D)

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Representative membrane associated signaling proteins and E) and F) Representative cytoskeleton associated proteins. Cell lysates (30 ug) were loaded onto each lane processed for SDS-PAGE and Western blotting procedure as described in Materials and Methods.

Fig. 4.

Differential expression of selected membrane, adhesion and cytoskeletal and regulatory proteins (A and B) in *in vitro* models of breast cancer progression: drug resistant and c-Jun-transformed MCF-7 cells. Comparison of expression profile with human normal fibroblasts WI38. Experimental conditions as in Fig.3

Fig. 5.

A) Effect of TSA on the expression of selected membrane, cytoskeletal and associated proteins in MDA-MB-231 cells. Cells were treated with 0.5 μM TSA for 48 hours. Cells were harvested and processed as described in Materials and Methods. Note that some Western blots were exposed much longer than shown in Figs. 1 to 3 which caused some proteins, such as cytokeratins, EBP50 or gelsolin to be detected in control MDA-MB-231 cells. Each experiment was repeated at least twice with similar result. B) Effect of TSA on the expression of adenosine receptor mRNA's determined by RT-PCR.

4 %

8 %

12 %

15 %

17.5%

20 %

22.5 %

25 %

35 %

4 hrs run
at 46 K

Lipid rafts (Fr. 1-4)

Soluble   (Fr. 5-6)
extract

Cytoskeleton (Fr. 7-10)

Chromatin (Fr. 10)

**Triton X-100 lysate loaded
in the middle of gradient**
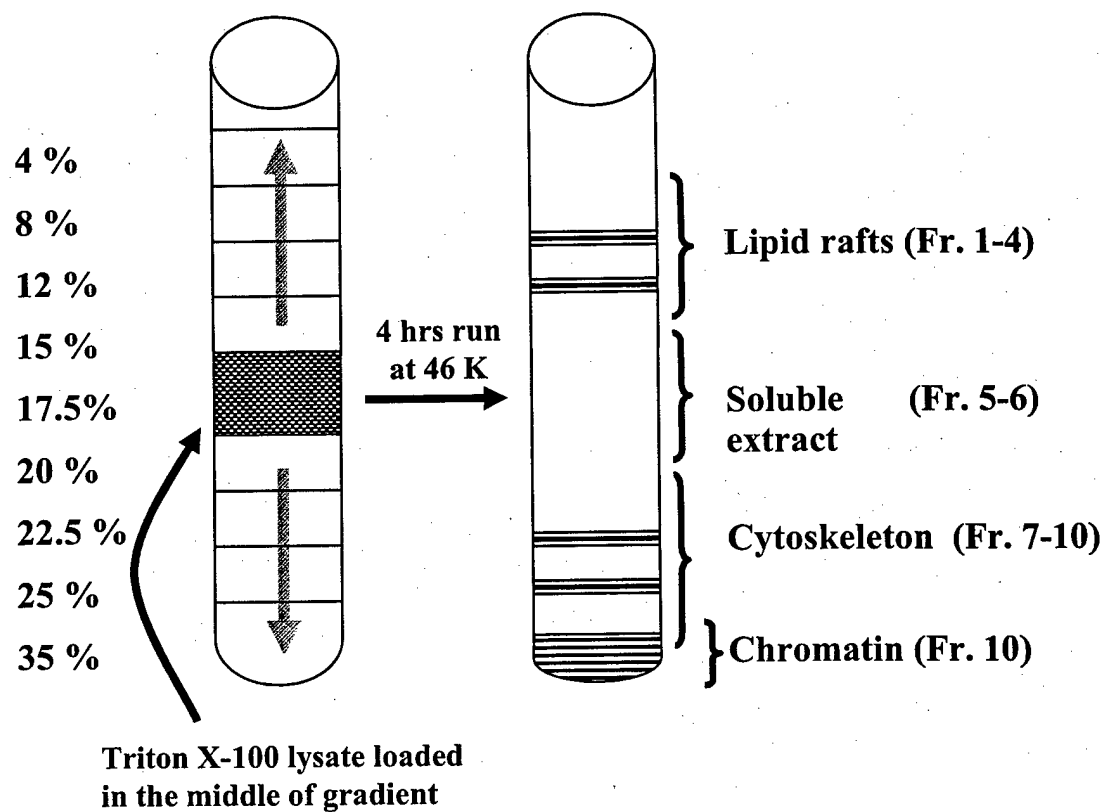
Fig. 1A, Ostapkowicz et al.

Density Gradient Fractions:
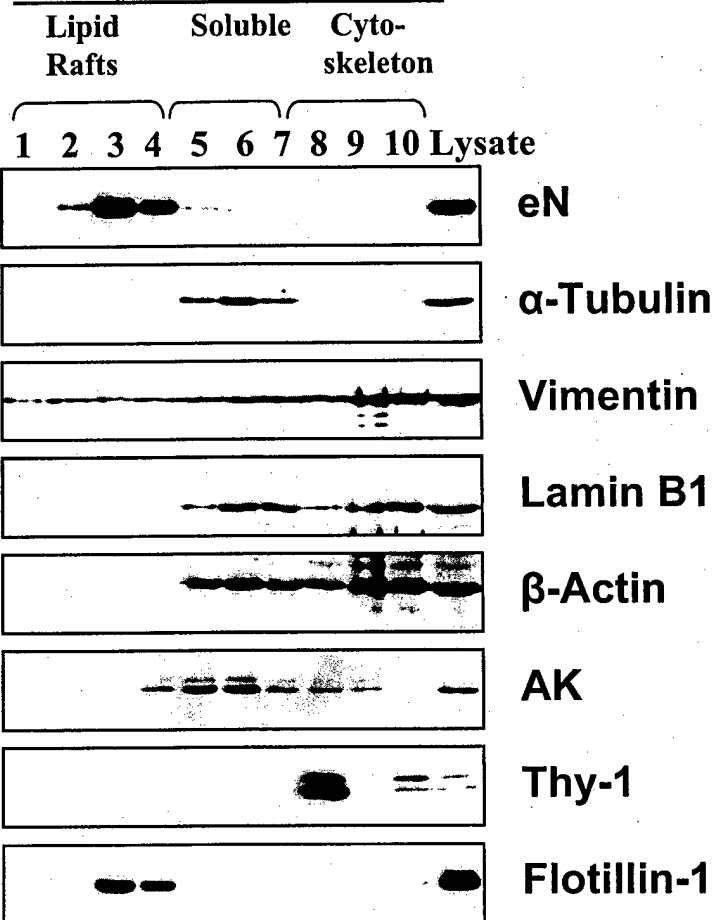
Fig. 1B, Ostapkowicz et al.
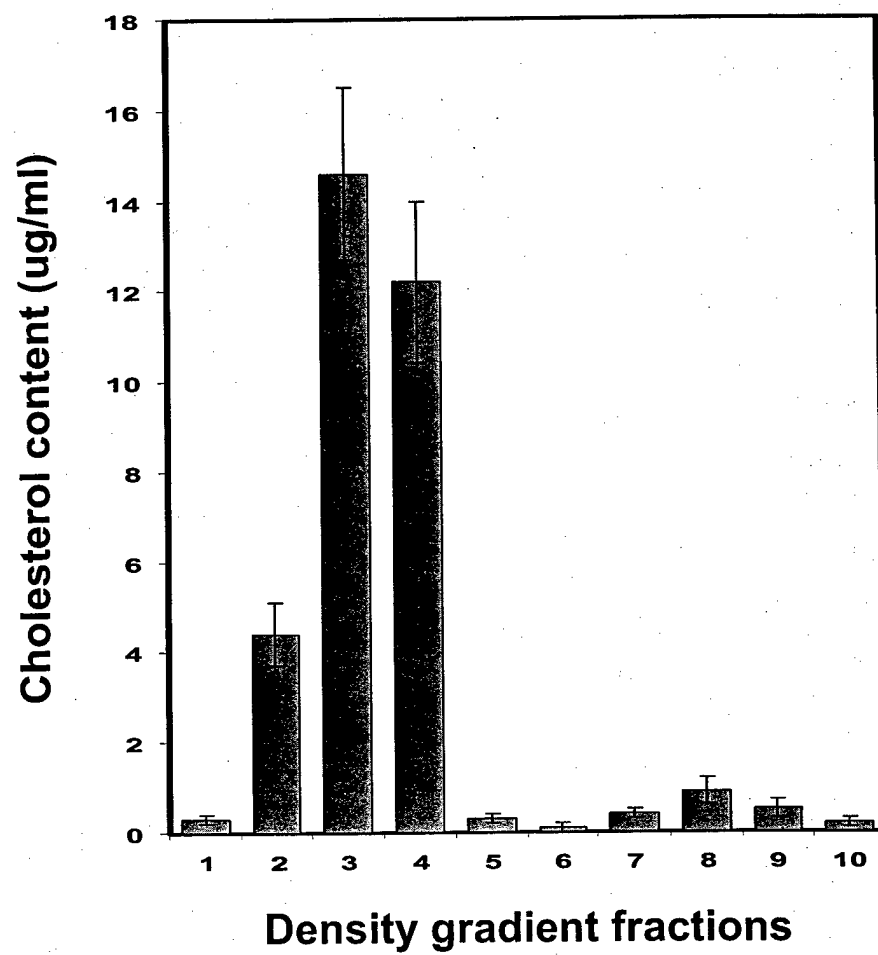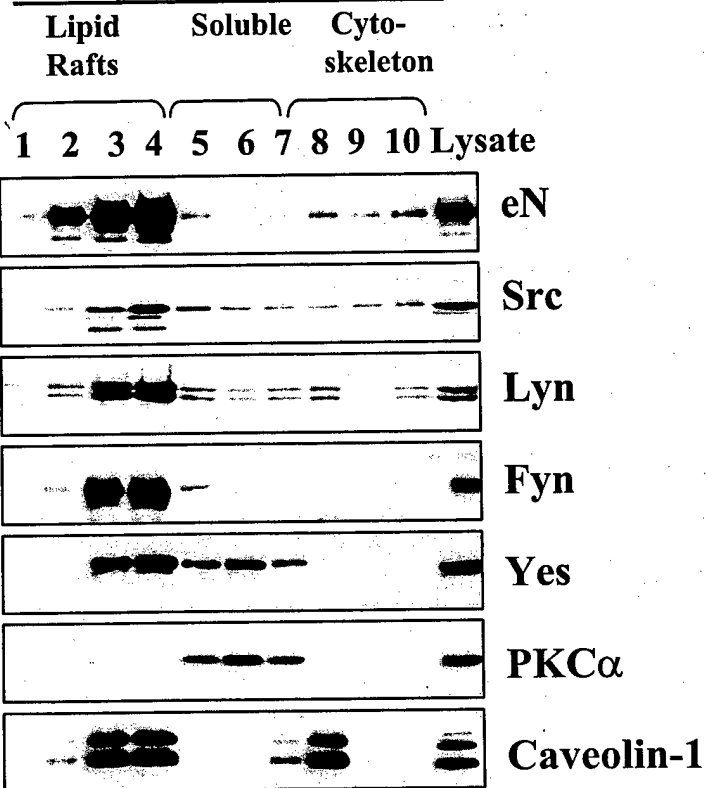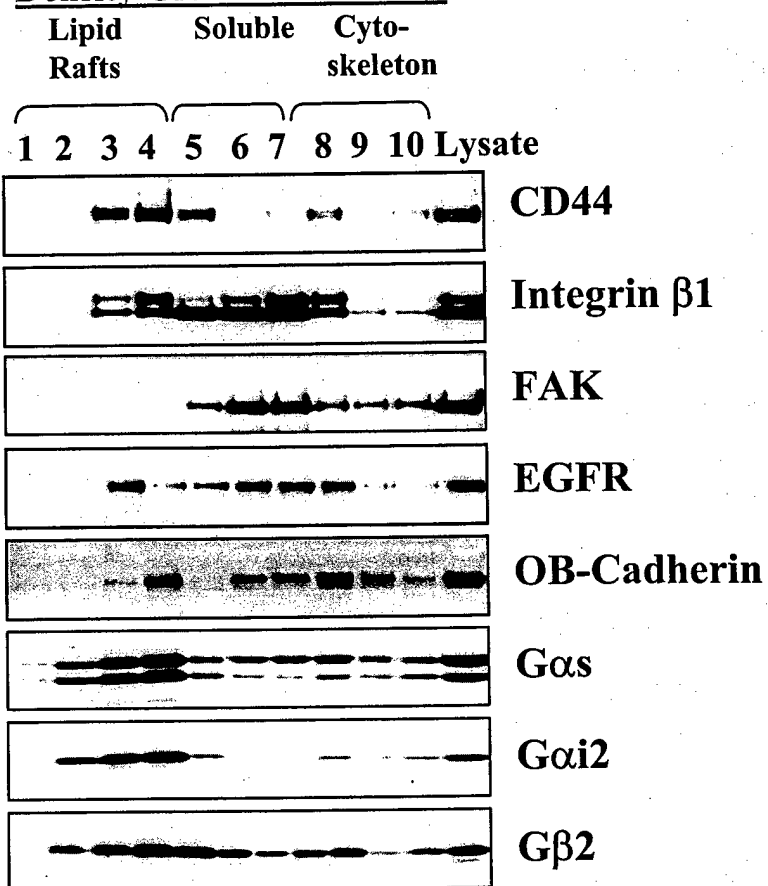
Fig. 1C, Ostapkowicz et al.

Density Gradient Fractions:

Lipid Rafts Soluble Cyto-skeleton

1 2 3 4 5 6 7 8 9 10 Lysate

eN

Src

Lyn

Fyn

Yes

PKCα

Caveolin-1

Fig. 2A, Ostapkowicz et al.

Density Gradient Fractions:

Lipid Rafts	Soluble	Cyto-skeleton

1 2 3 4 5 6 7 8 9 10 Lysate

CD44

Integrin β1

FAK

EGFR

OB-Cadherin

Gαs

Gαi2

Gβ2

Fig. 2B, Ostapkowicz et al.

Density Gradient Fractions:

Lipid Rafts   Soluble   Cyto-skeleton

1 2 3 4 5 6 7 8 9 10 Lysate

eN

CD24

Src

Lyn

Gas
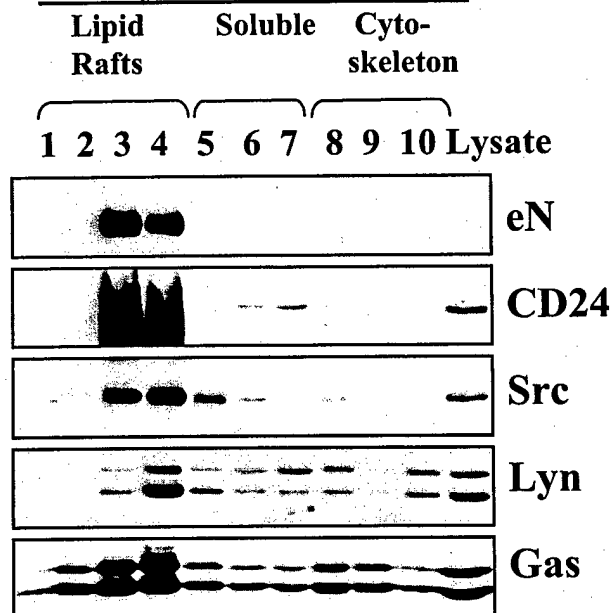
Fig. 2C, Ostapkowicz et al.

Fig. 3A, Ostapkowicz et al.
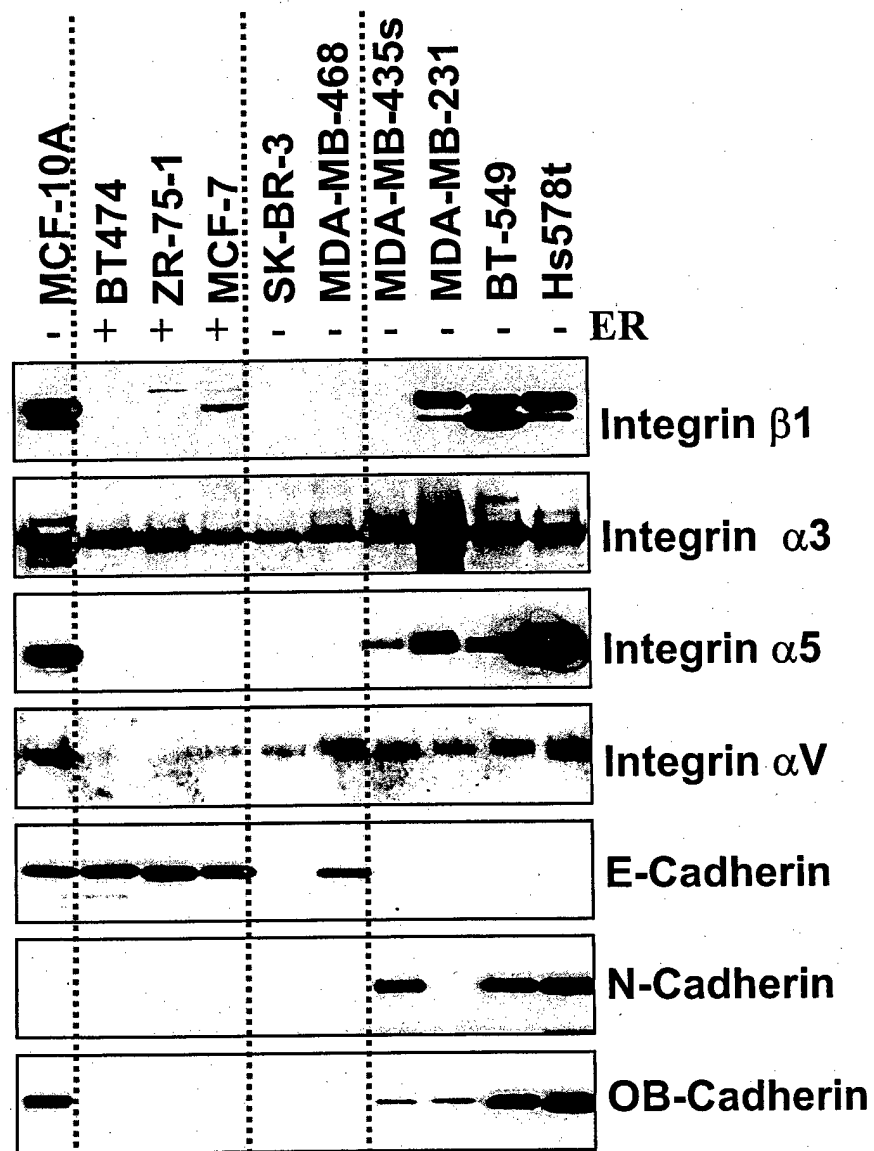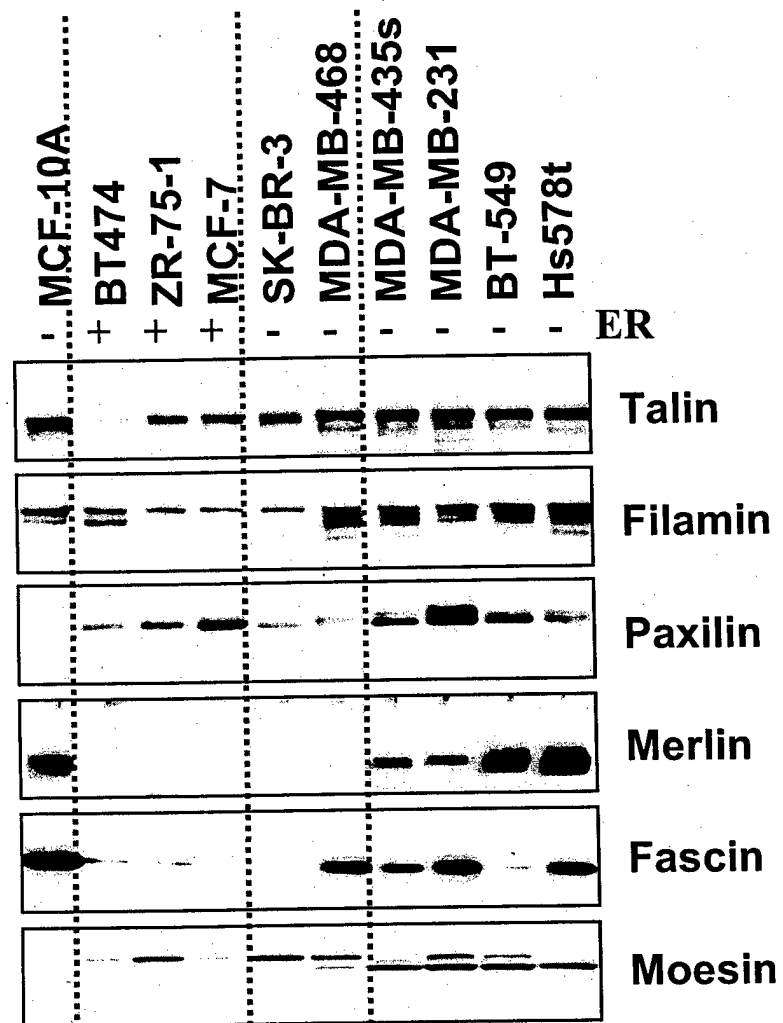
Fig. 3B, Ostapkowicz et al.
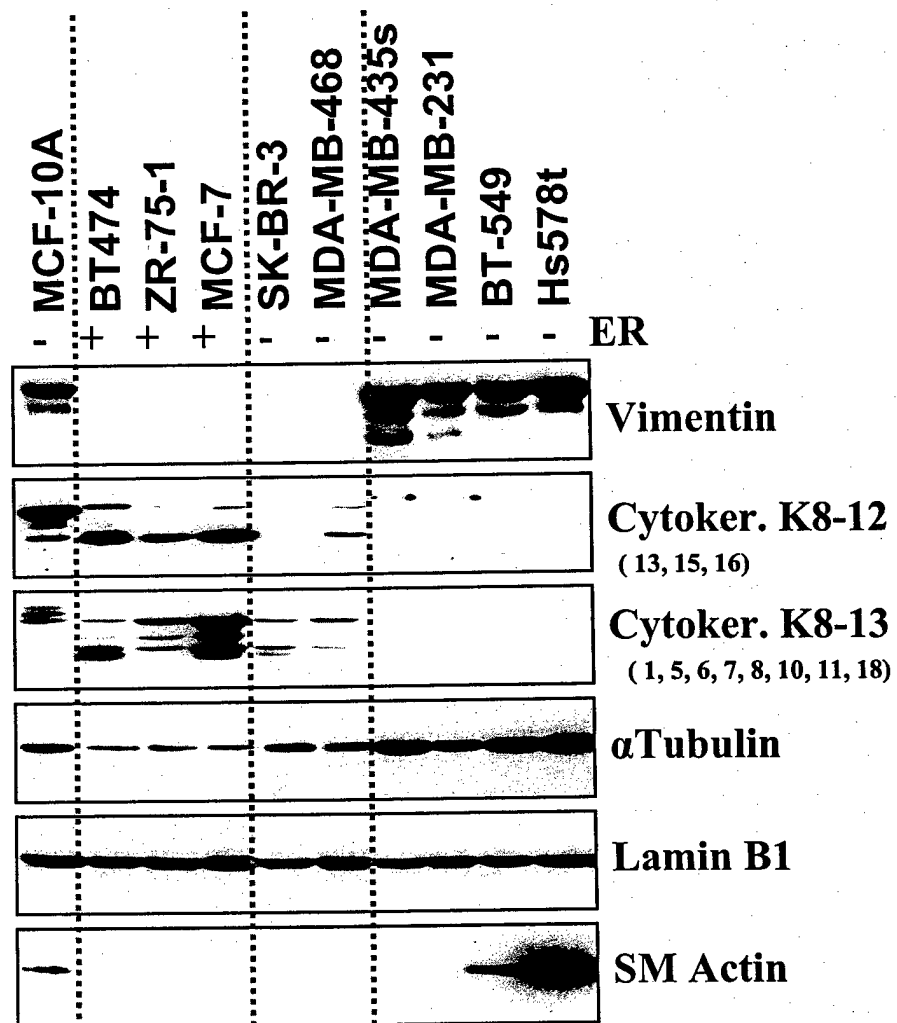
Fig. 3C, Ostapkowicz et al.
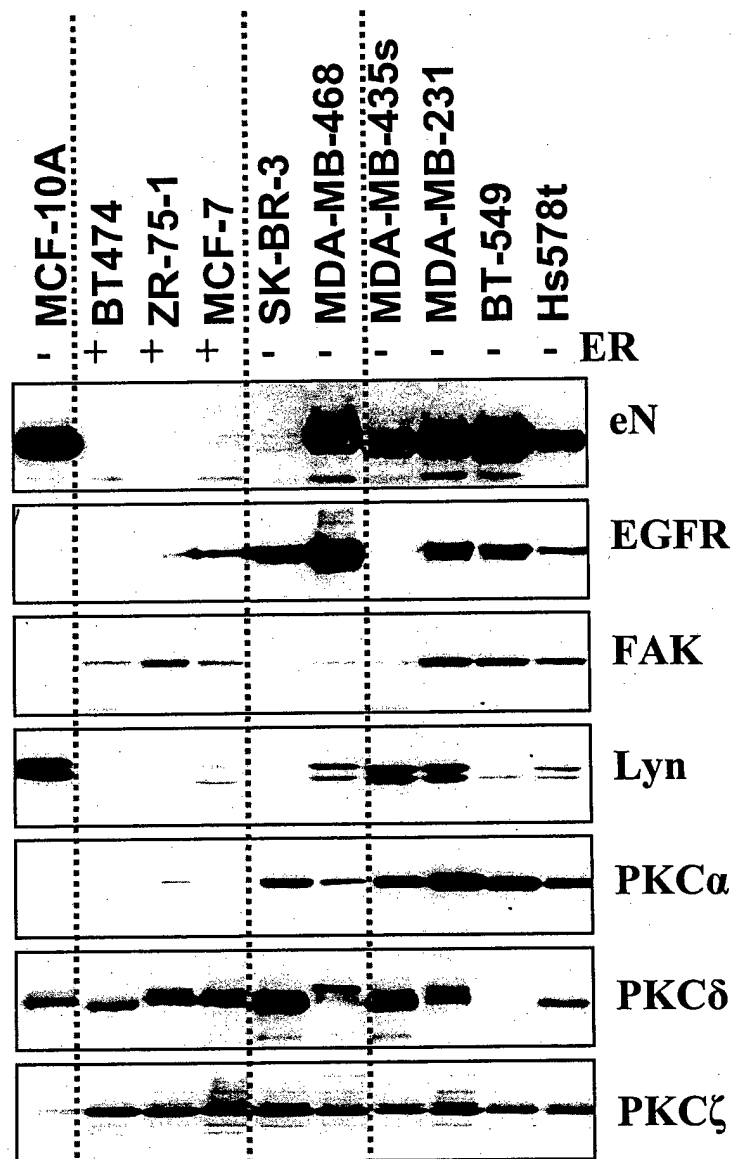
Fig. 3D, Ostapkowicz et al.
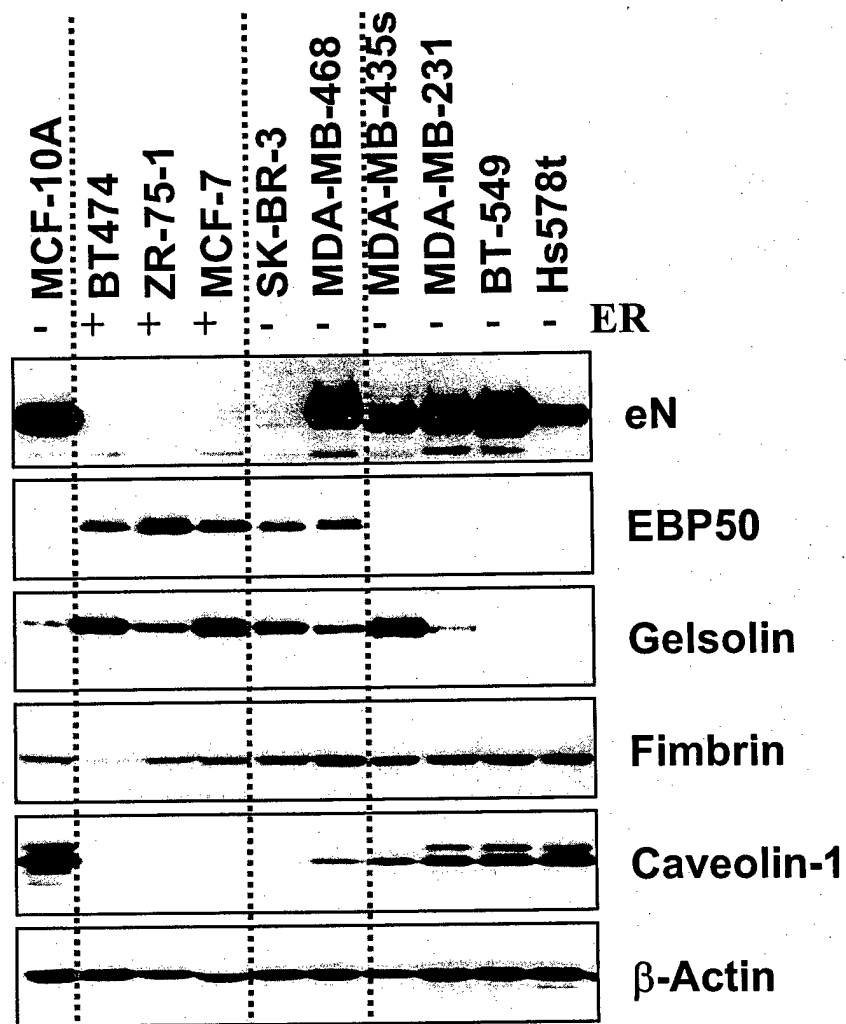
Fig. 3E, Ostapkowicz et al.
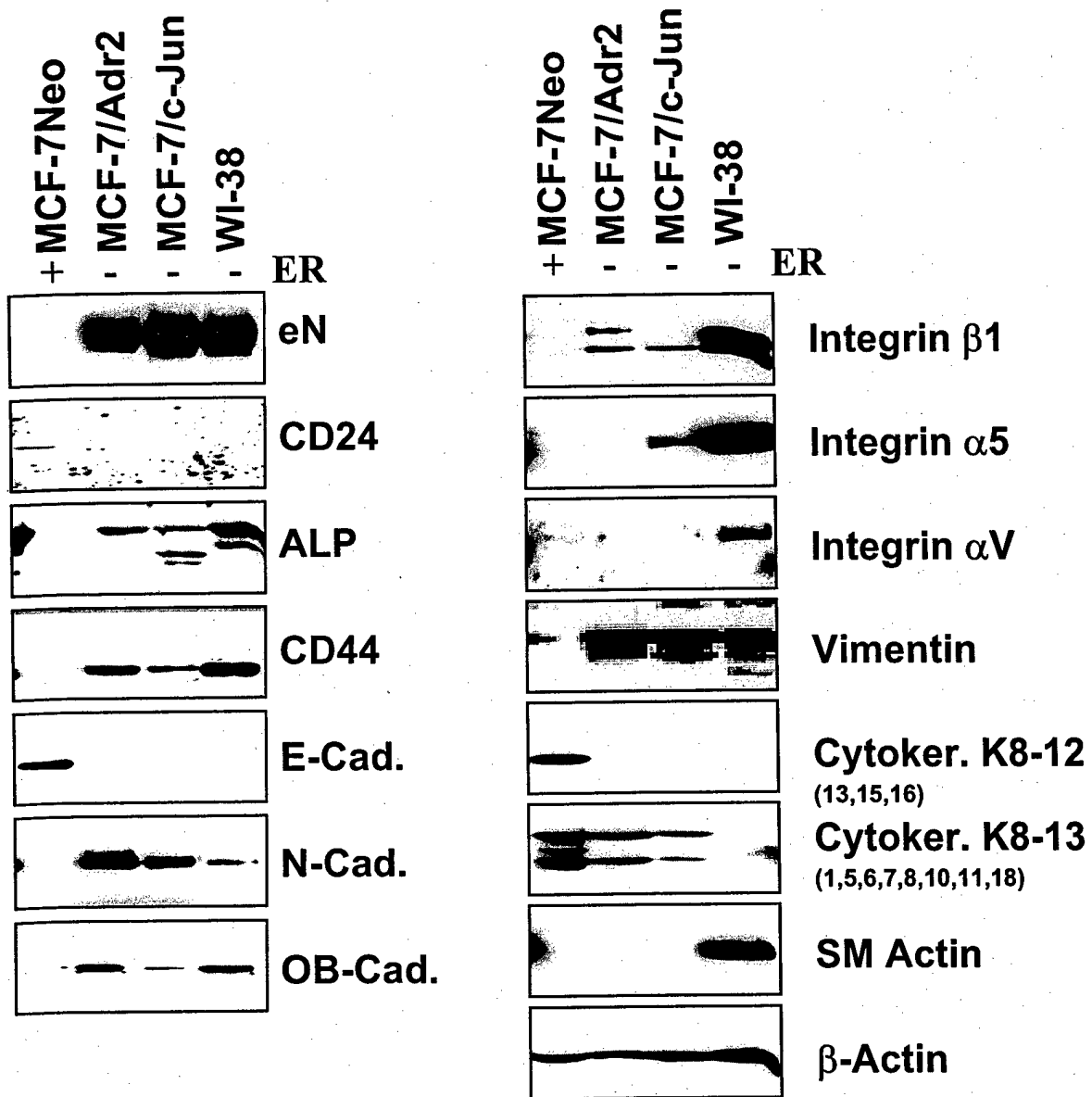
Fig. 3F, Ostapkowicz et al.
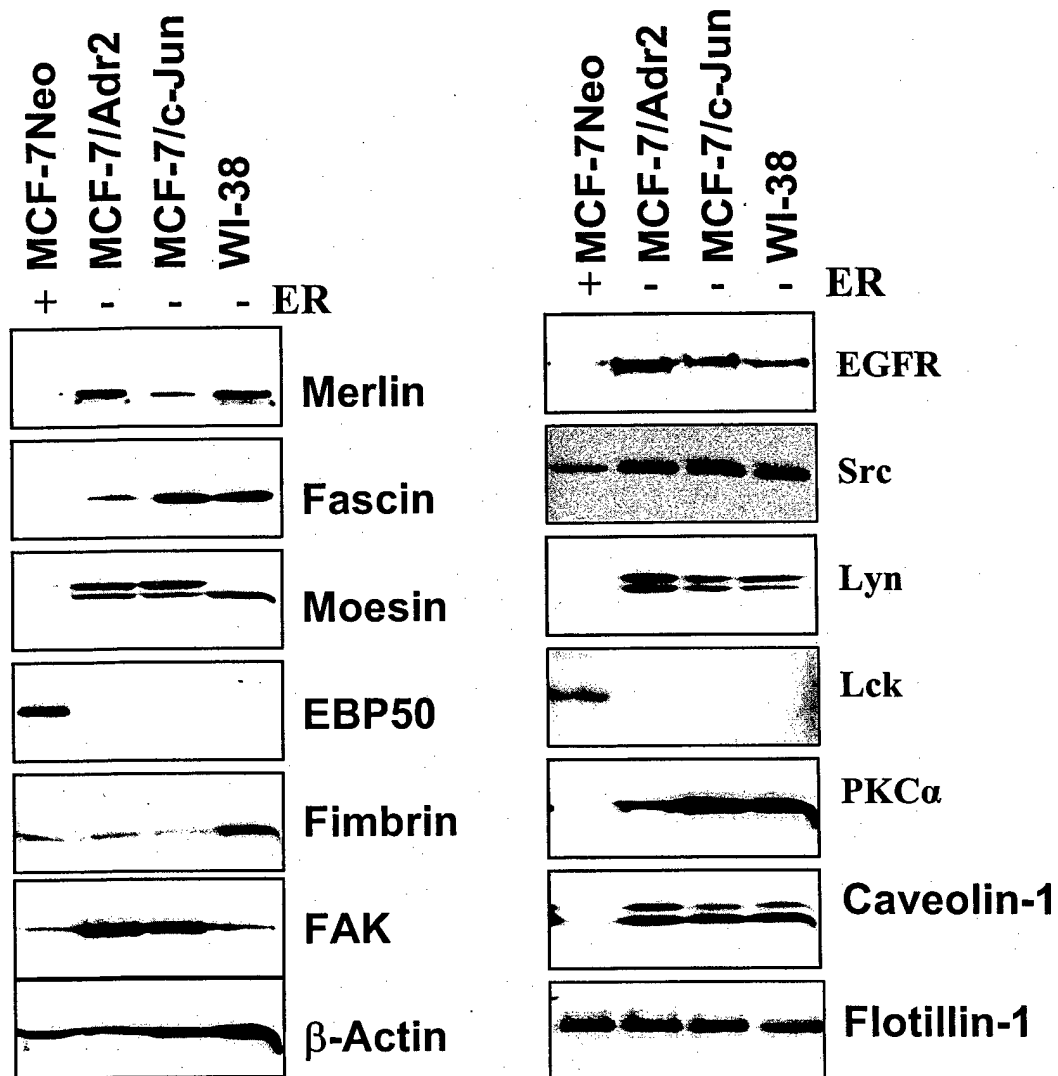
Fig. 4A, Ostapkowicz et al.
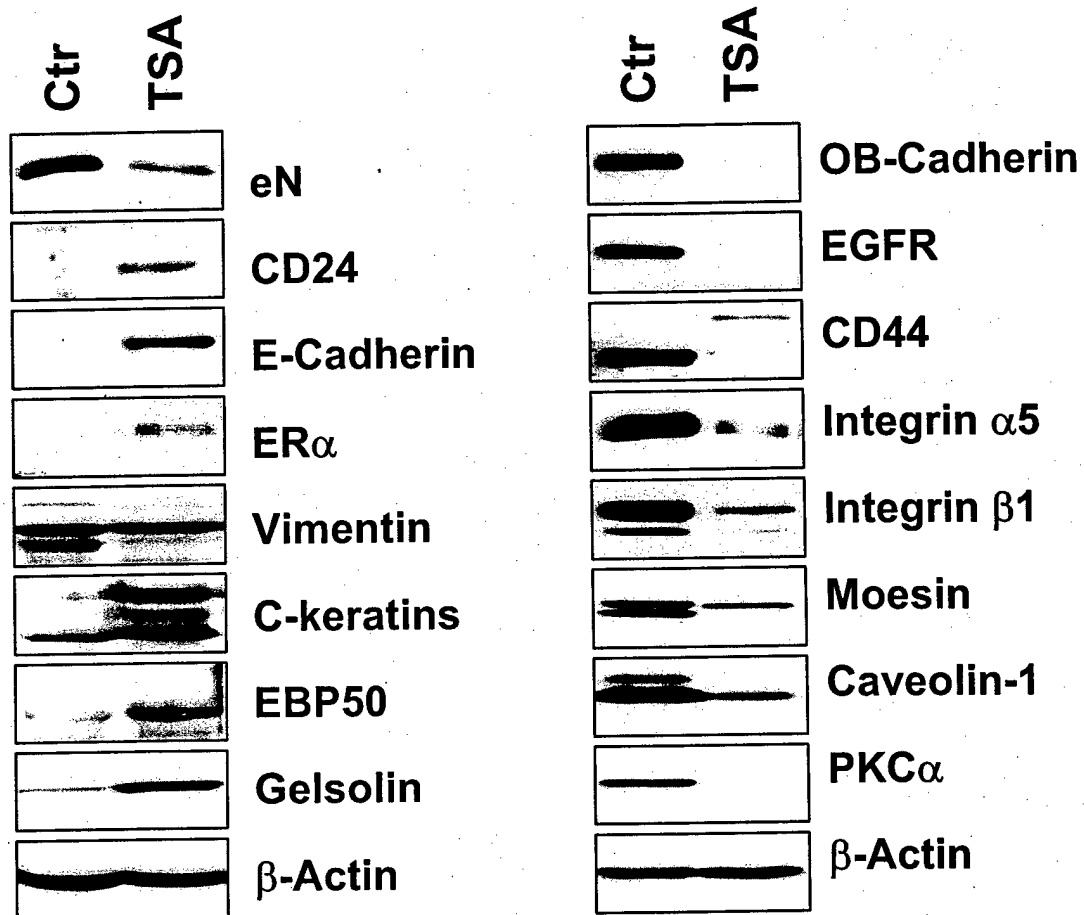
Fig. 4B, Ostapkowicz et al.
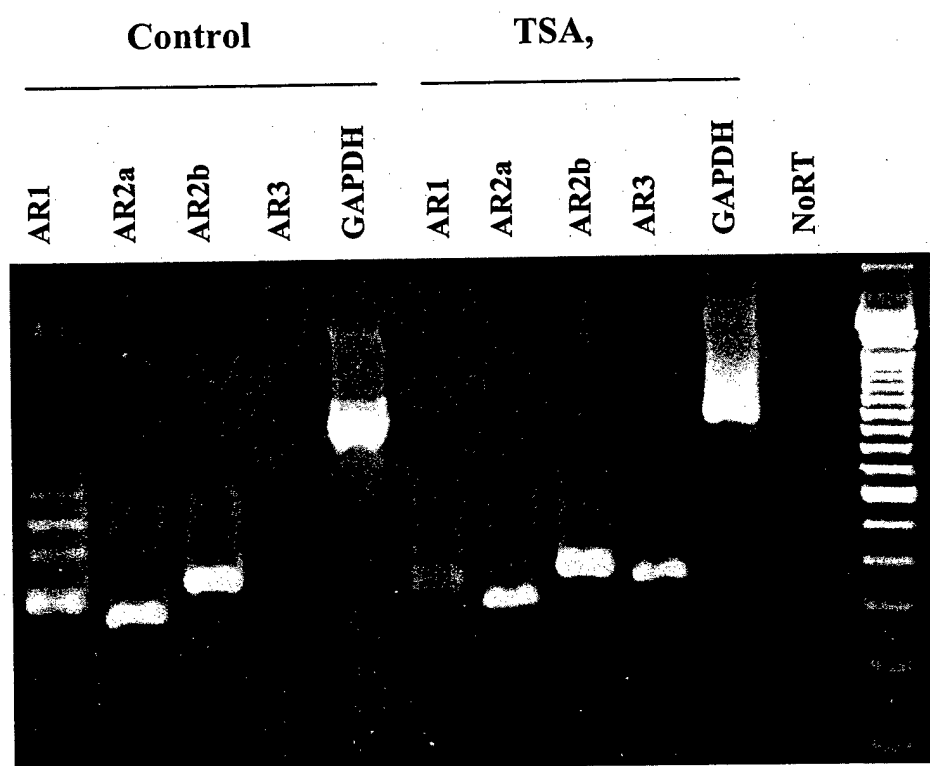
Fig. 5A, Ostapkowicz et al.

Control   TSA,

AR1   AR2a   AR2b   AR3   GAPDH   AR1   AR2a   AR2b   AR3   GAPDH   NoRT

Fig. 5B, Ostapkowicz et al.

Minireview

Mammalian 5'-Nucleotidases*

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Nucleoside monophosphate phosphohydrolases or 5'-nucleotidases (members of EC 3.1.3.5 and EC 3.1.3.6) dephosphorylate non-cyclic nucleoside monophosphates to nucleosides and inorganic phosphate. Seven human 5'-nucleotidases with different subcellular localization have been cloned (Table I). Sequence comparisons show high homology only between cytosolic 5'-nucleotidase IA (cN-IA)[1] and B and between cytosolic 5'(3')-deoxynucleotidase (cdN) and mitochondrial 5'(3')-deoxynucleotidase (mdN). However, the existence of common motifs suggests a common catalytic mechanism for all intracellular 5'-nucleotidases. Some 5'-nucleotidases are ubiquitous (ecto-5'-nucleotidase (eN), cN-II, cdN, and mdN); others display tissue-specific expression (cN-I and cN-III).

Here we summarize recent advances on the structure and cellular functions of the cloned 5'-nucleotidases. We also propose a revised nomenclature, agreed upon with other colleagues active in the nucleotidase field.

Catalytic Mechanism

Crystal structures are known for human mdN (10) and cdN[2] and for *Escherichia coli* periplasmic 5'-nucleotidase (11), a homologue of eN. All intracellular nucleotidases share a DX-DX(V/T) motif critical for catalysis and show structural similarity to the haloacid dehalogenase superfamily of enzymes (10). eN belongs to a separate family that includes also 2',3'-cyclic phosphodiesterases and apyrases (11).

The crystal structure of mdN and work on the active site of cN-II form the basis for a reaction mechanism of intracellular 5'-nucleotidases (10, 12). The reaction creates a phosphoenzyme intermediate involving the first aspartate in the DX-DX(V/T) motif (12). A detailed scheme of the catalytic process derived from the crystal structure of mdN (10) involved both aspartates in the above motif (Fig. 1). The first (Asp-41) generates a pentacovalent phosphorus intermediate with similar basic organization as the intermediate detected in the structure of β-phosphoglucomutase (13). The x-ray structure of cdN suggests a catalytic mechanism identical with that of mdN. Differences within the active sites account for differences in substrate specificity (10).[2] Using the structurally important

residues the best alignment was between the two deoxynucleotidases and cN-III (10). Two 5'-nucleotidases, cN-II and cN-III, exhibit phosphotransferase activity (for reviews see Refs. 14 and 15) possibly because of higher stability of the phosphoenzyme intermediate or faster exchange of the nucleoside product with the nucleoside acceptor.

The active site of *E. coli* 5'-nucleotidase, the paradigm for eN, contains two zinc ions and the catalytic dyad Asp-His (11). No phosphoenzyme intermediate is formed during catalysis, but a water molecule performs the nucleophilic attack on the phosphate (16).

Properties, Detection, and Inhibition of 5'-Nucleotidases

All 5'-nucleotidases have relatively broad substrate specificities. In agreement with the structural information on the active sites (10, 11), all family members except eN are absolutely dependent on magnesium for activity. Table II summarizes some distinctive properties of 5'-nucleotidases. Detection of individual nucleotidases by enzymatic assays in cell lysates is problematic because different nucleotidases are co-expressed in the same tissue or cell type. The problem was earlier addressed by immunotitration (for review, see Ref. 14) and more recently by a strategy that exploits differences in optimal conditions for the ubiquitous nucleotidases (17). Differential assays can take advantage of inhibitors of individual nucleotidases (8, 17–20). The most active inhibitors described so far are pyrimidine nucleotide and nucleoside analogs inhibiting cN-I at nanomolar or low micromolar concentrations with up to 1000-fold selectivity for cN-I relative to cN-II or eN (18). Two pyrimidine phosphonates inhibit cdN and mdN (8, 17) with weaker inhibition of cN-I (17). Specific properties of individual 5'-nucleotidases are discussed below.

Ecto-5'-nucleotidase—eN, also known as CD73, is a glycosylated protein bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor (1) and co-localizes with detergent-resistant and glycolipid-rich membrane subdomains called lipid rafts. Up to 50% of the enzyme may be associated to intracellular membranes (for review, see Ref. 20) and be released during homogenization. Early reports of a soluble low K_m nucleotidase (for review, see Ref. 20) were because of this phenomenon (21). Although eN has broad substrate specificity, AMP is considered to be the major physiological substrate (for review, see Ref. 20) (22–24). Independently of the enzymatic function, the protein acts as co-receptor in T cell activation (for review, see Ref. 23) and as cell adhesion molecule (for review, see Ref. 24). eN is variably expressed in a wide number of cell types under physiological and pathological conditions (for review, see Refs. 20, 23, and 24). In neuronal cells eN expression is linked to developing or plastic states (for review, see Ref. 24). The proximal promoter region of the gene contains a number of tissue-specific elements (25, 26).

Cytosolic 5'-Nucleotidase IA—cN-IA was named AMP-specific 5'-nucleotidase for its high specific activity with AMP at millimolar concentrations. Subsequent detailed kinetic studies revealed high affinities toward deoxypyrimidine monophosphates (18). It is highly expressed in skeletal and heart muscle where it has a physiological function in the generation of signaling adenosine during ischemia (2, 27). The high affinity for deoxynucleoside monophosphates suggests a role in the regulation of deoxyribonucleotide pools. A homologous sequence

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[1] The abbreviations used are: cN, cytosolic 5'-nucleotidase; cdN, cytosolic 5'(3')-deoxynucleotidase; eN, ecto-5'-nucleotidase; mdN, mitochondrial 5'(3')-deoxynucleotidase; NT, nucleotidase.

[2] A. Rinaldo-Matthis and P. Nordlund, manuscript in preparation.

TABLE I
Classification of 5'-nucleotidases

| Revised protein nomenclature	Full name and gene symbol	UniGene cluster no.	Aliases	Refs.
eN	Ecto-5'-nucleotidase, *NT5E*	Hs.153952	Ecto-5'-NT; low K_m 5'-NT; eNT; CD73	1
cN-IA	Cytosolic 5'-nucleotidase IA, *NT5C1A*	Hs.307006	AMP-specific 5'-NT; cN-I	2, 3
cN-IB	Cytosolic 5'-nucleotidase IB, *NT5C1B*	Hs.120319	cN-IA homolog; AIRP	4
cN-II	Cytosolic 5'-nucleotidase II, *NT5C2*	Hs.138593	High K_m 5'-NT; purine 5'-NT; GMP,IMP-specific 5'-NT	5
cN-III	Cytosolic 5'-nucleotidase III, *NT5C3*	Hs.55189	PN-I; P5'N-1; UMPH	6
cdN	Cytosolic 5'(3')-deoxynucleotidase, *NT5C*	Hs.67201	dNT-1; PN-II	7, 8
mdN	Mitochondrial 5'(3')- deoxynucleotidase, *NT5M*	Hs.16614	dNT-2	9
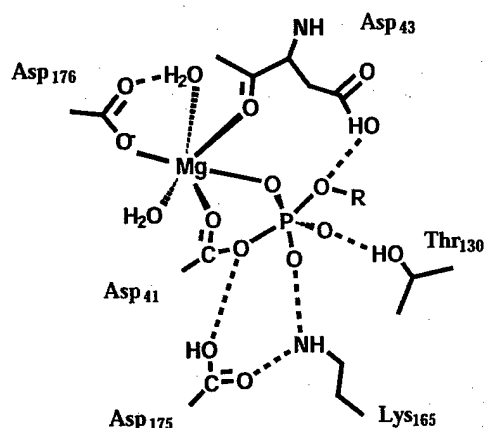
FIG. 1. **Structure of the active site of mdN with the pentavalent phosphorous intermediate produced by nucleophilic attack of Asp-41 on the phosphate (10).** Asp-43 first promotes the protonation of the leaving deoxyribonucleoside (*R*) and then activates the water nucleophile that releases the phosphate. Asp-41 and Asp-43 are the two aspartates in the motif conserved in intracellular 5'-nucleotidases.

related to human autoimmune infertility gene (*AIRP*) and with highest expression in testis has been cloned and designated cN-IB (4).

Cytosolic 5'-Nucleotidase II—cN-II is a 6-hydroxypurine-specific nucleotidase, most active with (d)IMP (for review, see Ref. 14) and critically positioned to regulate ATP and GTP pools. This tetrameric protein is stimulated by (d)ATP and GTP and regulated by substrate and phosphate in a complex manner (for review, see Ref. 14) (27–29), possibly involving subunit association and dissociation (30). Under physiological conditions cN-II can catalyze phosphotransfer from a purine nucleotide donor to inosine or guanosine (31, 32). This reaction is responsible for the activation of several anti-viral and anti-cancer nucleoside analogs that are not substrates for cellular nucleoside kinases (33, 34).

Cytosolic 5'-Nucleotidase III—cN-III is highly expressed in red blood cells where it participates in the degradation of RNA during erythrocyte maturation (for review, see Ref. 15). It prefers pyrimidine ribo- over deoxyribonucleotides with CMP being the best substrate. It is inactive with purine nucleotides. The enzyme has a phosphotransferase activity (35) less efficient than cN-II (32). The sequence of cN-III coincides with that of p36, an interferon α-induced protein of unknown function (6). Alternative splicing of exon 2 gives rise to two proteins that are 286 and 297 amino acids long (36), with the shorter form corresponding to cN-III.

Cytosolic 5'(3')-Deoxynucleotidase—cdN is a ubiquitous enzyme, first purified to homogeneity from human placenta (37). It is the major deoxynucleotidase activity in cultured human cells (17, 38). In contrast to cN-III, human cdN is not strictly pyrimidine-specific and works efficiently with dIMP and dGMP. dAMP is a poor substrate and dCMP is inactive (8, 37).

The enzyme is very active on 2'- and 3'-phosphates (7, 37). Neither the highly purified human placental cdN nor the recombinant mouse and human enzymes showed phosphotransferase activity (7, 37), in contrast to what was reported for cdN purified from human red blood cells (35).

Mitochondrial 5'(3')-Deoxynucleotidase—mdN is highly homologous to cytosolic cdN (52% amino acid identity). The two enzymes are coded by nuclear genes with identical structure, probably derived by a gene duplication event (9). With its high preference for dUMP and dTMP, mdN shows remarkably narrow substrate specificity. Similarly to cdN, mdN prefers deoxy- over ribonucleotides and accepts 2'- and 3'-nucleoside monophosphates (9, 38). Its enzymatic features suggest that mdN regulates mitochondrial dTTP and prevents accumulation of mutagenic dUTP within mitochondria.

Physiological Role of 5'-Nucleotidases: Insights from Overexpressing Cell Lines

By opposing the phosphorylation of nucleosides by kinases, intracellular 5'-nucleotidases participate in substrate cycles that regulate the cellular levels of ribo- and deoxyribonucleoside monophosphates and thereby all ribo- and deoxyribonucleotide pools (for review, see Ref. 39) (40). Intracellular 5'-nucleotidases have relatively high K_m values and operate on substrates generally present at (very) low concentrations. Thus they are exquisitely sensitive to oscillations of substrate concentration. Given their overlapping substrate specificities, it is difficult to tie a given enzyme to the maintenance of a specific nucleotide pool. Important information has been obtained with cell lines engineered to overexpress individual nucleotidases. Involvement of a 5'-nucleotidase in a specific substrate cycle is signaled by the increased excretion of the nucleoside produced by that cycle (40). In such experiments it is important to analyze the turnover of individual pools during brief time windows. Changes in nucleotide pool sizes only show the final end point of complex metabolic adaptations and may be a consequence of reduced ATP availability (29, 41). By this strategy cN-IA was shown to operate on AMP (27, 42) and cN-II on IMP and GMP (27, 40), and murine cdN was shown to regulate all pyrimidine deoxyribonucleotide pools (40). In human cells dCMP should be dephosphorylated by a different enzyme, as human cdN is inactive on dCMP. A potential candidate is cN-IA that has high affinity for all deoxyribonucleotides (3, 18), although it is still not clear whether the expression of this enzyme outside skeletal and heart muscle is sufficient to perform this function (3). Strategies such as knockout mice and small interfering RNA may help solve these issues. Indeed, down-regulation of mdN in cultured human cells by small interfering RNA showed that mdN participates in a mitochondrial substrate cycle with the mitochondrial thymidine kinase.[3]

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[3] C. Rampazzo and V. Bianchi, unpublished data.

TABLE II
Distinctive features of 5'-nucleotidases

Enzyme	Protein structure (monomer kDa[a])	Substrate affinity (K_m)	Effect of ATP	pH optimum
eN	Dimer (63)	μM	–	7.5
cN-IA[b]	Tetramer (41)	μM–mM[c]	+ (ADP ++)[d]	7
cN-II	Tetramer (65)	sub mM	++	6.5
cN-III	Monomer (33)	sub mM	none	7.5
cdN	Dimer (23)	mM	none	6–6.5
mdN	Dimer (26)	sub mM	none	5.5

[a] Predicted from cDNA sequence and not including posttranslational modifications.
[b] cN-IB not yet characterized.
[c] Micromolar K_m values for pyrimidine deoxynucleotides; millimolar or submillimolar for purine substrates (3, 18).
[d] ADP and dADP are best activators (S. A. Hunsucker and Y. Spychala, manuscript in preparation).

Clinical Implications of 5'-Nucleotidases

The only known genetic syndrome due to 5'-nucleotidase deficiency is the hereditary hemolytic anemia caused by mutation of cN-III (for review, see Ref. 15). Accumulation of normally undetectable pyrimidine nucleotides in erythrocytes of affected subjects highlights the important role of cN-III during maturation of red blood cells.

Anti-viral and anti-blastic nucleoside analogs must be activated by phosphorylation to exert their therapeutic effect. Intracellular 5'-nucleotidases influence the metabolism of the analogs by reversing the activation step and thereby decreasing their pharmacological efficacy. Several of the *in vitro* models of nucleoside analog resistance were linked to high expression of cN-II and cN-IA (3, 43) (for review, see Ref. 44). Consistent with the substrate cycle model, the relative ratio of nucleoside kinase to 5'-nucleotidase may have predictive clinical value with 5'-nucleotidases contributing to drug resistance (45). Development of compounds that inhibit 5'-nucleotidase activity may reverse drug resistance and increase the efficacy of existing analogs. New nucleoside analogs that are poor substrates for 5'-nucleotidases may lead to more effective therapies. Several anti-viral drugs show striking mitochondrial toxicities that pose a serious limitation on their use (46). One way to address this problem is to develop compounds that are differentially metabolized by cytosolic and mitochondrial 5'-nucleotidases, thus allowing for cytoplasm-specific accumulation of pharmacologically active metabolites. Recent development of cdN and mdN inhibitors that differentially target these enzymes suggests that such strategy is possible (17).

Conclusions

The presence in the human genome of at least seven genes for 5'-nucleotidases suggests that these enzymes perform important metabolic functions. With the enzymes available in recombinant form it will soon be possible to complete their biochemical characterization. Gene regulation remains instead an unchartered field. We do not know how strict is the tissue-specific expression of cN-III, how the variable expression levels of the ubiquitous nucleotidases revealed by multiple-tissue Northern blots are obtained, if expression of individual enzymes can be induced or repressed in specific circumstances, and if patterns of expression are altered in specific tumors.

The new RNA technologies and microarray analyses of overall expression profiles in cells will contribute to clarify the role of 5'-nucleotidases in the regulation of nucleotide pools. We are looking forward to the new information to come.

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